UTILIZATION OF DAIRY WASTEWATER FOR SUSTAINABLE PRODUCTION

A THESIS

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"What is great in man is that he is a bridge and not an end […]."

Friedrich Nietzsche

Abstract

Inefficient use of nitrogen and phosphorus leads to anthropogenic eutrophication of rivers, lakes, and oceanic basis worldwide, causing an environmental problem that can trigger a death cycle of an entire water body. Microalga, one of the organisms that benefits from the excessive nutrient runoff and uses the sunlight to catalyze reactions that cause eutrophication, can also be part of a solution. The study presented in this thesis represents a possible integrated solution for the nutrients accumulated, especially in dairy farms. This work shows how it is possible to treat dairy wastewater in large volumes, using plastic photobioreactors with an initial inoculum of microalgae, in this case *Chlorella sp.*, in a mixotrophic solution (since the dairy wastewater used in this study was not sterilized). It was also shown that in pilot-scale, ratio 1:10 (dairy wastewater in water) was capable of removing high amounts of nutrients, up to 97.55% of ammonium, 39.27% of nitrate, and 27.05% of phosphate. The 1:10 was also capable of producing competitive biomass amounts when comparing to the controls, 1.575 ± 0.599 $g.L^{-1}$ and 1.315 ± 0.240 $g.L^{-1}$. Moreover, none of the treatments (control, controlN, 1:10, 1:10N, 1:30 and 1:30N) were significantly different from each other, considering the nutrients (NH_4^+, PO_4^{3-}, NO_3^-) removal rates and biomass, when adding or not extra CO_2. In addition, a study was carried to evaluate the taste preference of calves fed *Chlorella sp.* produced in the previous steps. Sterilized biomass was used for feeding trials with six Holstein and crossbred dairy heifer calves. No mycotoxins were found in the biomass and many heavy metals were tested, having the levels below the maximum content recommended for animal feeding. The microalgae biomass produced had a protein content of 49.2%, 2.32% of fat, 38.5% of

carbohydrates, and around 10% of different minerals and nutrients. They were fed 0, 30, and 60 g of *Chlorella sp.* daily in a sequential elimination study. No difference were found for dry matter intake of calves fed 0, 30, or 60 g of *Chlorella sp.*, indicating that microalgae may be added to the rations of calves without any adverse effects.

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Chapter 1

Project Introduction

1.1. Problem Description

Minnesota is one of the top ten milk production states in the United States, with a total of 822 million pounds of milk produced between May and June of 2019 (USDA, 2019). Every year 3.8 billion dollars are generated by the dairy industry in Minnesota and the industry generates approximately 9,500 jobs (MDA, 2016). Minnesota-known as the land of 10,000 lakes-has many freshwater sources including over 10,000 lakes and the Mississippi River which passes through the state and drags nutrients all the way to the Gulf of Mexico. The nutrient accumulation, in particular nitrogen and phosphorus, are produced mainly by the addition of fertilizers on crops and by the manure from livestock. These nutrients can cause severe eutrophication problems in water all around the world.

Eutrophication is a process caused by the excessive discharge of inorganic and organic nutrients of nitrogen and phosphorus (NOAA, 2017). During the eutrophication process, due to the high levels of nutrients present, various populations like bivalve mollusk and algae proliferate (Bennett et al, 2001; Tilman, et al, 2001). Algae blooms are involved in biofilm formation at the water surface, which may impede the sunlight to transpose it, causing the death of plants that are in the lake or ocean bed. The death of these plants may cause organic matter accumulation and oxygen (O_2) depreciation. However, the plants will die as the oxygen level decreases. Bacteria and other organisms that feed on the organic matter will drive, increasing its population and releasing gases, like carbon dioxide (CO_2)

and methane (CH_4). Depending on the size of the water body, and with a higher possibility of occurrence in lakes (because the water does not move a lot), the O_2 concentrations can became defficient and kill most of the animals and plants underwater. In addition, the increase of CO_2 will decrease the pH and turn the water into a more acidic environment (Glibert et al., 2005; Rabalais, et al., 2009; Moss, 2011).

Dairy wastewater usually is abundant in milk, fats, washing detergents (used to clean the milking facility) and nutrients (nitrogen – in the form of nitrates, ammonium and nitrite, phosphate, sulfur and organic carbon). These nutrients are usually applied directly on croplands as a fertilizer (Cheung & Wong, 1981; Daneshvar et al., 2019). Therefore, combining microalgae properties that remove nutrients from dairy wastewater and biomass growth may result in environmental and economic solutions to help Minnesota farmers improve water quality, by recycling nutrients and producing a potential high protein product for livestock feed.

1.2 Overview of a Possible Solution

Considering eutrophication as one of the biggest problems related to water resources nowadays in the United States, especially in Minnesota, with all the water resources that it has (MDH, 2017). Action needs to be taken concerning the development of a technology that farmers can apply on their properties, cleaning wastewater, reducing the nutrients application in the soil, but using these nutrients as a raw material to produce added-value products that can be used in the farm or can be sold.

Dairy wastewater may be an excellent medium for the production of microalgae biomass, which can be used as livestock feed or biofertilizer. Microalgae that remove nutrients from dairy wastewater may be turned into a livestock feed if grown in bioreactors and removed from the water before the treated effluent final disposal (back to a water body or croplands). Adaptable to different ecosystems, microalgae need at least three key components to grow: sunlight, a carbon source and a moisturized environment (Rizwan et al., 2018). All of these elements can be found on farms (Daneshvar et al., 2019). Responsible for about half of the world atmospheric oxygen production (Singh et al., 2011), microalgae naturally use carbon dioxide (CO_2) from the atmosphere as a carbon source, water (H_2O) as a source of electrons, oxygen, and sunlight as the catalyst of the photosynthesis reaction.

In an effort to minimize eutrophication it is possible to treat dairy wastewater using *Chlorella sp.* (Hena et al., 2015) removed 98% of the nutrients from dairy wastewater using algae. Daneshvar et al., (2019) reported the removal of more than 92% of the total nitrates and 100% of the phosphate from raw and recycled dairy wastewater. Lu et al. (2015) used *Chlorella sp.* to remove nutrients from raw dairy wastewater using bioreactors of 40 Liters, and they were capable of producing 110mg/L/d of microalgae biomass and remove up to 65.33% of the total phosphorus, and total nitrogen depleted up to 85.17%.

The microalgae biomass has many applications and because of its high nutritive content it can be used for animal feed. Numerous studies have shown that *Chlorella sp.* has high crude protein content and can vary between 35 and 48% (Becker, 2007; Yaakob et al, 2014; Kang et al, 2013; Yan et al, 2012). Erickson et al. (2011) fed calves with different concentrations of kelp in their regular starter grain to test the palatability because kelp

would be an option for cattle feed supplementation. However, the authors found differences in the dry matter intake (DMI) between the treatment without kelp and the treatments with kelp and concluded that calves preferred the control (without kelp) over the treatments with kelp. Heins and Chester-Jones (2015) also used kelp as a livestock feed and reported that kelp might not be economically benefic since the cost of adding kelp would be 11.9% higher than the control grain.

Humans have used *Chlorella sp.* for many years as a supplement. However, because of the high production cost, it is not used currently for animal feed. Using a bioreactor may be a way to recycle nutrients from dairy wastewater and to produce *Chlorella sp.* biomass to run a taste preference study, optimizing the production of this microalgae and offering an option to farmers in Minnesota.

1.3 Aims

This thesis is the summary of the work completed to evaluate the production of microalgae biomass using dairy wastewater to conduct livestock studies. The goals of this project were:

(i) To use a well-adapted microalgae strain to the Minnesota environment to conduct lab-scale experiments with different ratios of dairy wastewater, measuring nutrient removal rates, growth capacity and biomass productivity by the microalgae during lab-scale experiments;

(ii) Design a pilot-scale bioreactor and a monitor/controller system for pH and temperature;

(iii) Use the results from the lab-scale to conduct pilot-scale experiments with different ratios of dairy wastewater to optimize microalgae growth and nutrient removal rates in an outdoor bioreactor system. Measure nutrient removal rates, and growth capacity for biomass productivity by the microalgae during pilot-scale experiments;

(iv) Evaluate the necessity of adding CO_2 as an extra source of carbon and pH regulator to optimize microalgae growth;

(v) Conduct palatable livestock feeding studies in calves using *Chlorella sp.* biomass produced in the previous steps of this study

1.4 Outline of Technical Content

In the following chapters of this thesis, research will be conducted by treating dairy wastewater using *Chlorella sp.* to recycle nitrogen and phosphorus, producing microalgae biomass that can be used as livestock supplement feeding. The microalgae used in this study was isolated from a dairy wastewater lagoon at the WCROC, in Morris, where the pilot-scale experiments and the taste preference study with calves were conducted. Due to the unique characteristics that dairy wastewater can have, lab-scale experiments were conducted to observe the behavior of the microalgae when exposed to light for long periods of time, constant aeration and carbon dioxide addition (Chapter 3; Lu et al 2015; Shi et al, 2016).

The lab-scale experiments elucidated ideas to design bioreactors for a pilot-scale trial (Chapter 2). The bioreactor design considered a future application on farms in Minnesota. They had to be reasonably low weight, easy to transport (they could be used inside of a greenhouse during the winter months), easily operated by anyone, low-cost maintenance, and effective on cleaning wastewater and growing microalgae. To help manage the reactions and understand what was happening inside the bioreactors, a control system was installed to monitor pH and temperatures changes, and also to control independently each one of the different dilutions used in each one of the experiments.

Furthermore, the lab-scale experiments were replicated in the pilot-scale to determine how the environment and different volumes would impact the biomass production and nutrient removal rates. All the pilot-scale experiments were conducted outdoors (Chapter 3; Lu et al 2015). Radiation, temperature and pH were monitored. The pH was controlled during the different dilutions (wastewater in different water ratios) experiment using liquid CO_2 cylinders. Furthermore, a study was conducted on pilot-scale to evaluate the difference in biomass production when adding extra CO_2 (Chapter 3; Ramaraj et al 2015; Liu et al 2017).

Being able to treat dairy wastewater with *Chlorella sp.* and produce enough biomass to use this microalga as a livestock feeding would solve many problems on farms. However, before scaling the production up, it would be helpful to test how the animals would accept eating *Chlorella sp.* Considering many studies made with kelp (Erickson et al, 2011; Yan et al, 2012; Kang et al, 2013; Yaakob et al, 2014), this study will feed calves with three different treatments (two of them using *Chlorella sp.* as supplement) to test the palatable acceptance by the animals (Chapter 4).

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Chapter 2

Bioreactor Design, pH and Temperature Monitoring System, and Harvesting Techniques

2.1 Lab-scale Bioreactors

The lab-scale bioreactor is composed of one box made of glass (height = 0.48m, length = 0.76m, width = 0.31m) opened on top, with an acrylic support for eight glass tubes (diameter = 0.075m, height = 0.05m) positioned in parallel to each other (Figure 1). Rubber stoppers were used to keep the system closed. A glass tube (diameter = 3mm) passing through the rubber stopper was used to inject air into the system and CO_2, when needed.

The medium used during lab-scale experiments (wastewater, AM6 and DI water) was not sterilized, so there was no need for air filters to be used. Each one of the systems had one blower injecting air at a rate of 0.8 L/min ± 0.1 L/min in each one of the tubes, adjusted by the flowmeters on the top left of Figure 2. The pH and temperature were monitored using the Apex system. Utilizing the Apex system, made it was possible to control the pH changes using a CO_2 gas cylinder and maintain pH for the algae to grow.

The CO_2 was added without being diluted, but was mixed with air before going into the glass tube and inside the reactor. The pressure was 17kPa (16psi) on the way out of the cylinder. The lights used during the lab-scale experiment were bought from Philips, model F28T5/830 ALTO, warm white, 2900 lumens, and 28 watts. The lab-scale experiments took place at the United States Department of Agriculture – Agricultural Research Service

(USDA-ARS) – Soils Management Research, at Morris, Minnesota. Figure 2 is an example of the set up used during the lab-scale experiments.

2.2 Pilot-scale Bioreactors

The pilot-scale bioreactors were designed to have six bags in one system, where all of them could receive approximately the same amount of solar radiation during the day. The structure was made using treated pinewood (height = 1.73m, length = 2.06m and width = 1.22m). Three pars of flexible metal bars (diameter = 0.05m, height = 1.34m), distant 0.03m from each other, were installed to support the shape of the hanging bags (Figure 4). PVC tubes (diameter = 0.015m, height = 1.33m, length = 0.52m) were used to blow oxygen into the bags and carbon dioxide, when needed. At the bottom of each PVC tube, twelve holes (diameter = 0.5mm) were drilled for the air to flow, aerating and mixing the whole system.

The bioreactors (pallet 1 and pallet 2) were set up outside of the dairy barn at the West Central Research and Outreach Center (WCROC) in Morris, Minnesota (Figure 4). Each one of the pallets had a box attached in one of the sides, containing the Apex system. A liquid CO_2 tank was used as a carbon source and to control the pH when needed. Each one of the bioreactors (two total) had a capacity for six hanging bags with a maximum volume of 80L each.

The airflow was measured using an 8360 VelociCalc® Plus from TSI, and it was flowing in a rate of 4.61 ± 0.09 m^3/min from the blower into the bag through the larger

black hose (this rate was measured in each one of the bags individually). Figure 5 has the detail of the air flowing system, where the smallest black hose (diameter = 0.48cm) was used to add carbon dioxide, and the larger black hose (diameter = 3.5cm) was responsible for inserting air from the blower into the bag. To not insert 100% CO_2 (CO_2 was added only during specific experiments) into the system, ideally the two fluids were mixing while entering together at the beige PVC tube and then flowing into the algal system.

2.3 Temperature and pH Monitoring and Controlling System

The Apex Fusion® consists of an electronic system created by the Neptune company (California) to monitor different parameters in aquariums. In this project, the Apex Fusion® was used as part of a temperature and pH monitor system and a pH controller system. Using Figure 6 as a reference, the items bought from Neptune are: the energy bar ("b"), the probes' modules ("g"), and the Apex controller ("f"). The main objective of using this system was to use the pH probes from the Apex Fusion® system to determine what was the pH and the temperature in each one of the bags.

Using the pH information, it was possible to program the Apex Fusion® to turn on the solid states relay (Figure 6, "c"), opening a valve from the manifold (Figure 6, "d") and releasing CO_2 automatically in each one of the bags when the pH reached a maximum value (8.6) and then closed the valve when the pH reached the minimum value (7.4). In order to make it possible, some electric devices have been added to the system (Figures 6 and 7).

The electric system in Figure 7 was specifically designed to include six valves (because each one of the bioreactors had six bags) using a manifold (VALV). The manifold made it possible to have independent addition of CO_2 into the bags. However, the manifold is 24V AC, and the Apex Fusion® system is 120V AC, which means that an extra switch had to be added to the system. In order to make it possible, the solid state relays (RLY) were added to turn on and off the manifold, and they were 120V in one side (to connect to the energy bar) and 24V in the other side (to be able to connect to the manifold). The transformer was added to maintain the manifold energized with 24V in one side, and to connect the manifold to the 24V side of the RLY. All the RLY's, probe modules, the transformer, and the Apex Fusion® controller were connected to the energy bar, and the energy bar was connected to the line.

In summary, the monitoring and controlling system measures temperature and pH and are saved in the Apex Fusion® controller (Figure 6, "f"). When the pH probe has a measurement over 8.6 it will inform the Apex Fusion® controller to unlock the designated switch and turn on the solid state relay (RLY) for the specific bag. The RLY will open one of the manifold valves, and the valve will release CO_2 inside the bag until the pH probe gets a measurement below 7.4, then the Apex controller will lock the switch, turning off the RLY and the valve.

2.4 Solar Radiation and PAR Monitoring

During the lab-scale, in addition to controlling and monitoring the pH, and monitoring the temperature, it was possible to use the Apex Fusion® system to monitor the

photosynthetic active radiation (PAR) that the microalgae culture was receiving from the lights. A special probe module (ASM) was added to the system, and a PAR sensor was added to it. The photosynthetic active radiation refers to the visible light that ranges between 400nm and 700nm, and the unit used is µmol/m^2/s.

During the lab-scale experiments, the PAR sensor was positioned in between tubes, to measure the photosynthetic active radiation that the tubes were receiving and it varied between 331 and 338 µmol/m^2/s. Furthermore, the lights were set up to be on every day between 5 a.m. and 9 p.m. to imitate the duration of the sunlight received by the bags during the pilot-scale when they were outside during the summer.

During the pilot-scale, the bioreactors were positioned outside and exposed to ambient changes between July and August. The orientation of bags was east to west. The solar radiation during pilot-scale was measured using the data from the United States Department of Agriculture – Agricultural Research Service (USDA-ARS),that have permanent experiments going on near Swam Lake, which is approximately 11km from the West Central Research and Outreach Center (WCROC). The information provided by the USDA-ARS was an average of the radiation for the day and ranged between 40.56 and 303.55 W/m^2. To be able to compare lab-scale and pilot-scale, an approximate conversion was used, where, for sunlight conversion, 1 µmol/m^2/s was equal to 4.57 W/m^2 (Langhans, R.W., et al., 1997), so the solar radiation received by the bags on pilot-scale varied between 185.34 and 1387.23 µmol/m^2/s. A clear sky during the summer solstice in the northern hemisphere, at noon, would have a maximum photosynthetic active radiation of 2000 µmol/m^2/s (Slaterry et al, 2017).

2.5 Harvesting Techniques

The lab-scale experiments were not harvested because of the small amount of biomass produced. In that case, for the lab-scale, only the biomass production per day was evaluated filtering 15ml of the sample through a 0.22µm filter paper. However, for the pilot-scale, two different harvesting techniques had to be used. Furthermore, it was essential to quantify the total biomass produced, especially for the experiments where biofilm formation was observed. The harvesting technique used for the bags with different dilutions (when wastewater was diluted in water) and controls bags (AM6 medium and water) had to be different because of the time management and centrifuge size in use at the laboratory.

The *Chlorella sp.* used in this study had different behavior when dealing with dairy wastewater than when diluted in artificial medium only. A uniform distribution of microalgae cells was observed in the entire water column (70L) when using AM6 medium and having the blower turned off for more than 24h. However, when using wastewater and water, the microalgae tend to precipitate easily after turning off the blower between 3 and 5 hours.

2.5.1 Harvesting *Chlorella sp.* from Artificial Medium in Pilot-scale

When harvesting the biomass from the pilot-scale experiments, the blower was turned off after the last sampling. The microalgae biomass was expected to precipitate, accumulating in the bottom of the 70L bag. However, what was observed for the controls,

was that the microalgae would stay distributed in the entire water column and only a minimal amount going to the bottom of the bag. Therefore, the entire bag had to be centrifuged. A milk cream electric centrifugal separator (version 100-18), designed by Motor Sich JSC©, was used to centrifuge the algae biomass. The initial capacity of this product was to separate 100 L in one hour with 75 rpm. However, to separate microalgae, some modifications had to be made, including not using the twelve disks that are supposed to be inside the centrifuge. For this project, the microalgae would stay trapped inside the centrifuge, and the liquid part would leave the separator system. Because of that, every time that the water leaving the system started to be dark green, the separator was turned off, and the biomass was harvested (Figure 10) from inside the centrifuge system. After collecting the biomass, it was sterilized under 121°C and a pressure of 15 psi, for 30 minutes. The biomass was then kept frozen at -4°C after drying in an incubator for eight days at 65°C.

2.5.2 Harvesting *Chlorella sp.* from Different Dilutions of Dairy Wastewater in Water in Pilot-scale

After taking the last sample of each experiment, the blower was turned off, making it possible for the microalgae biomass in the bags containing dairy wastewater, to precipitate after a minimum of 3 hours. In this case, less than five liters of the mixture of water and wastewater containing *Chlorella sp.* biomass had to be centrifuged using a Sorvall Legend XTR Centrifuge, from Thermo Scientific, at 6500*xg* during 2 min. The biomass was collected, sterilized under 121°C and a pressure of 15 psi, during 30 minutes.

The biomass was then kept frozen at -4°C after drying in an incubator for eight days at 65°C.

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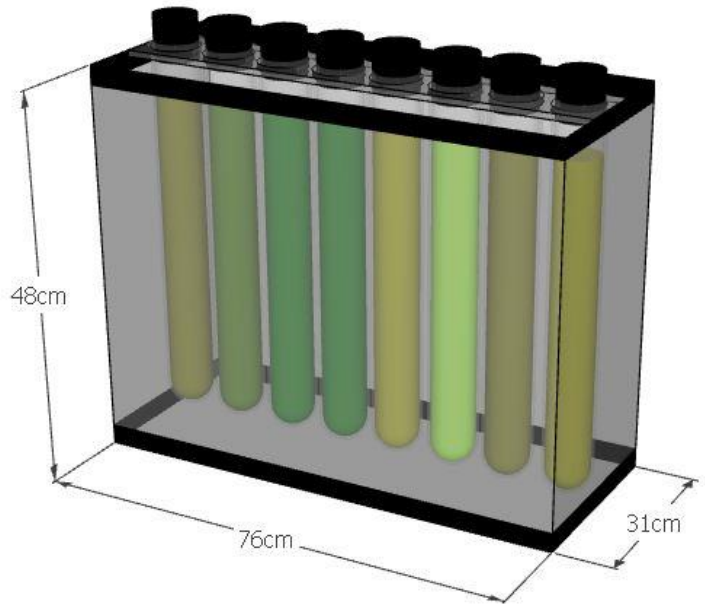

Figure 1. Glass tubes used during the lab-scale experiments.

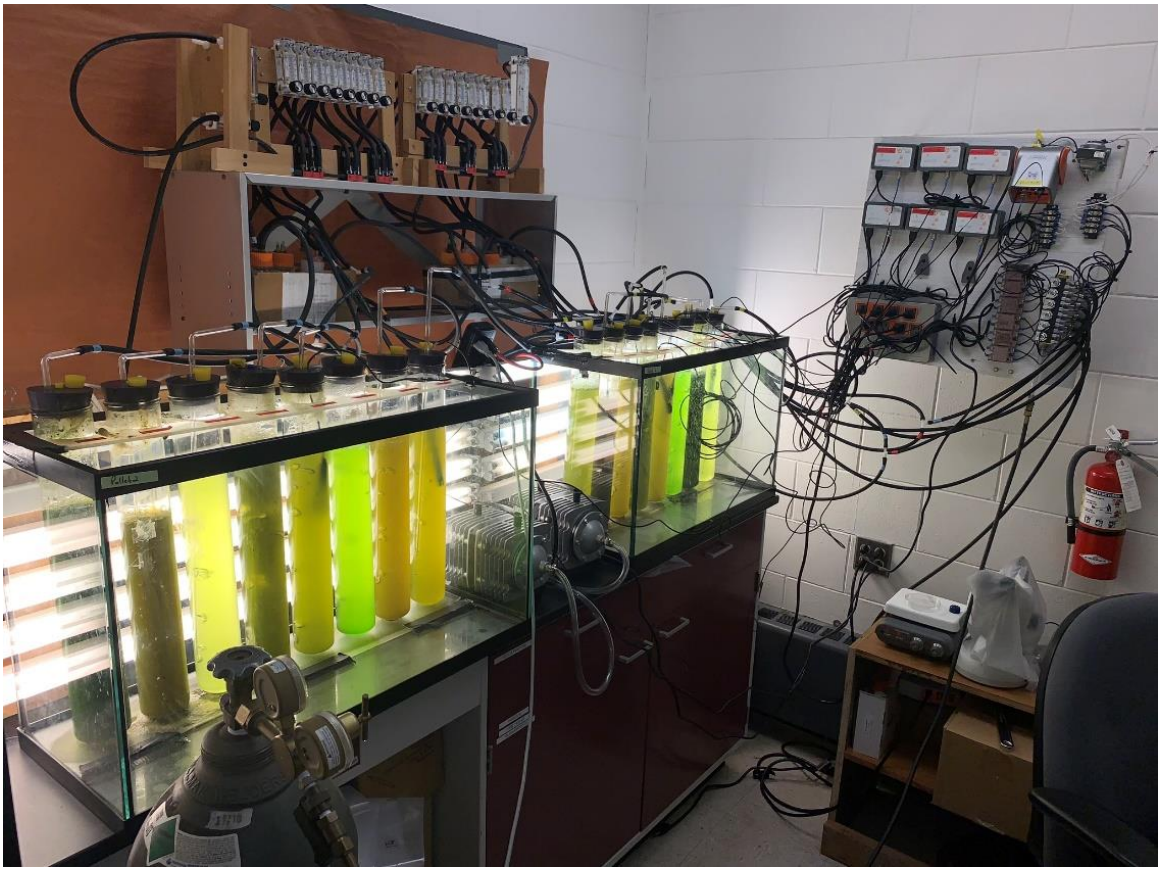

Figure 2. Experimental set up during lab-scale.

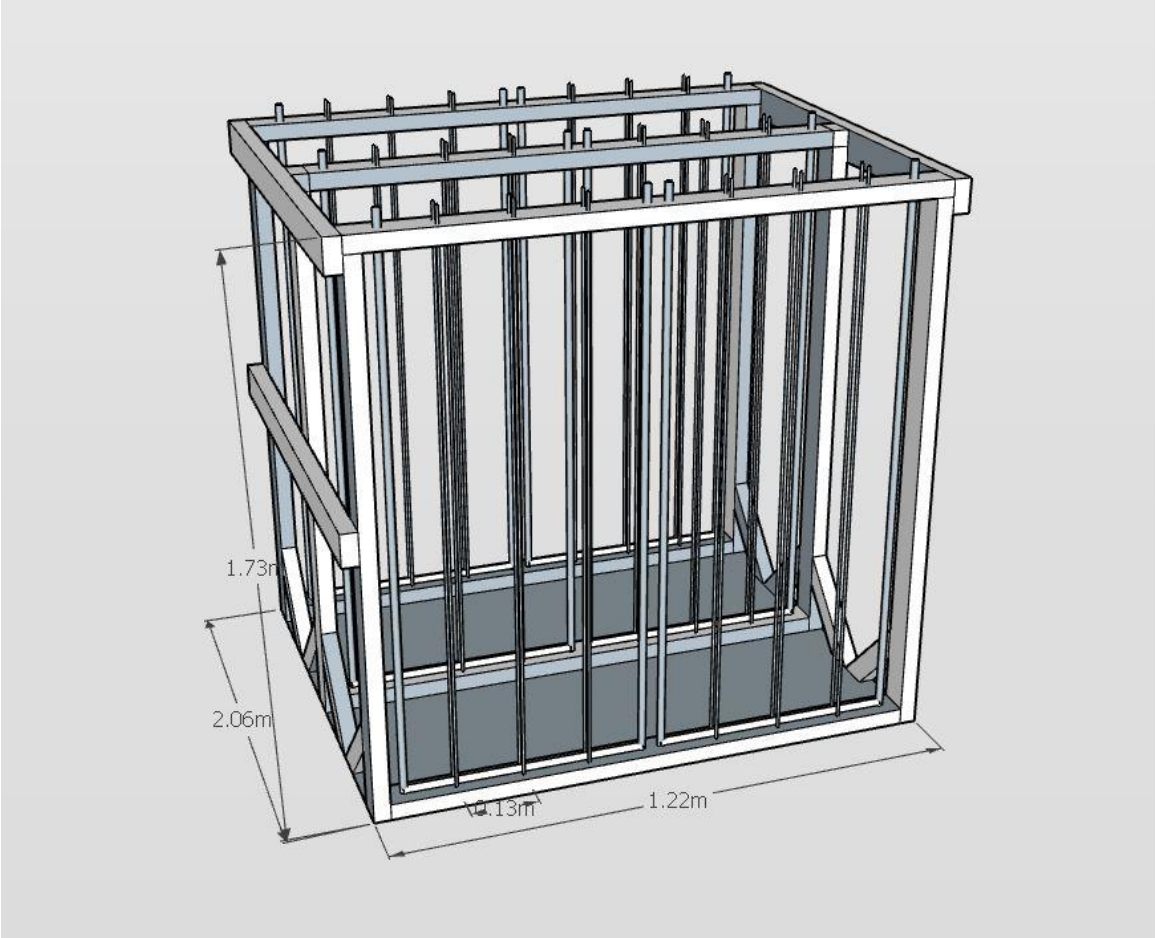

Figure 3. Sketch of the pilot-scale bioreactors.

Figure 4. Pilot-scale set up outside of the dairy barn at WCROC – Morris, Minnesota, during the summer.

Figure 5. Detail on the air and carbon dioxide mixture going into the system.

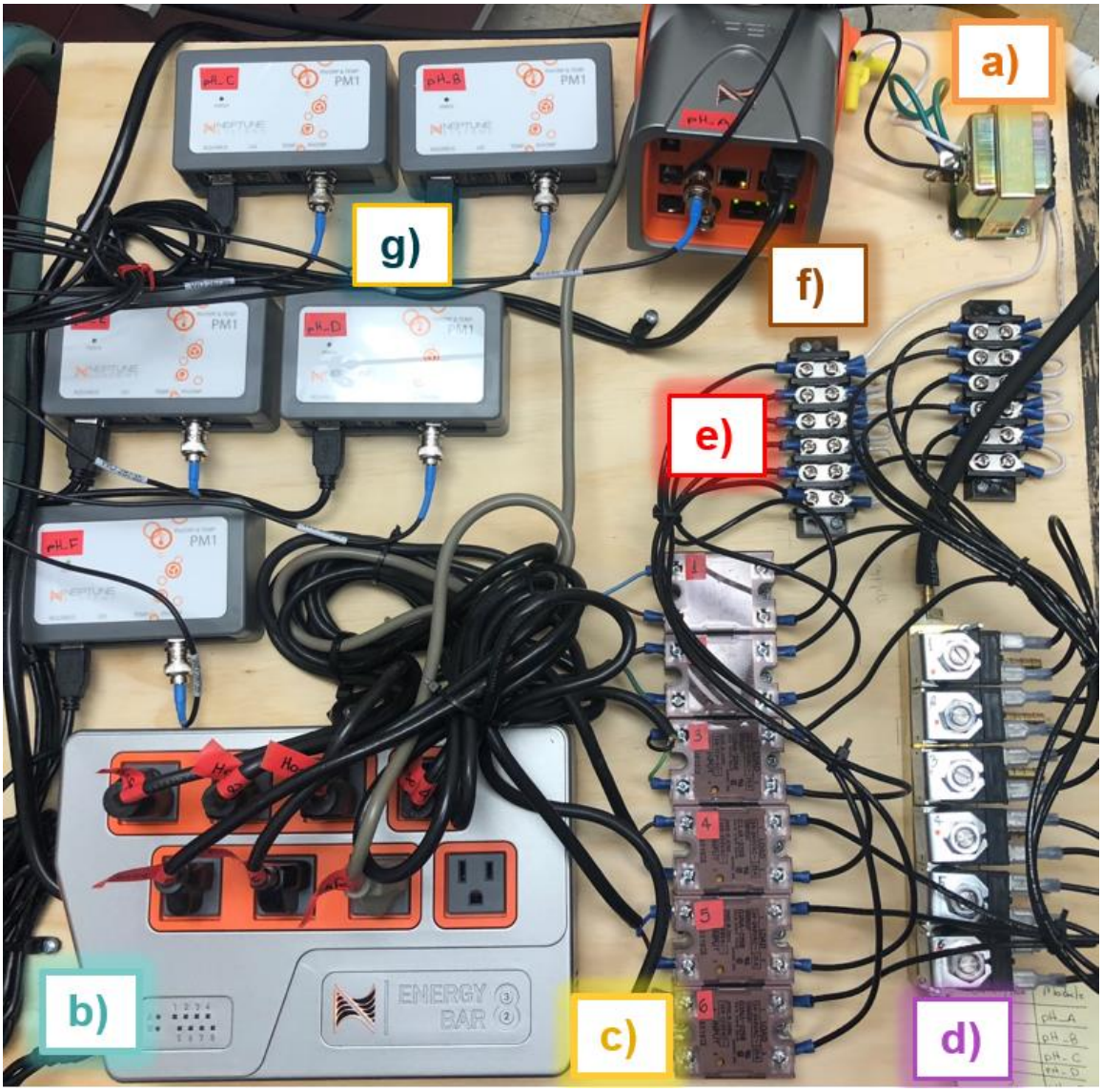

Figure 6. Temperature and pH monitoring and controlling system, where “a” is the transformer, “b” is the energy bar or switch, “c” are the solid state relays, “d” is the manifold or valves, “e” is the energy splitters, “f” is the Apex Fusion® controller, and “g” is the probe modules.

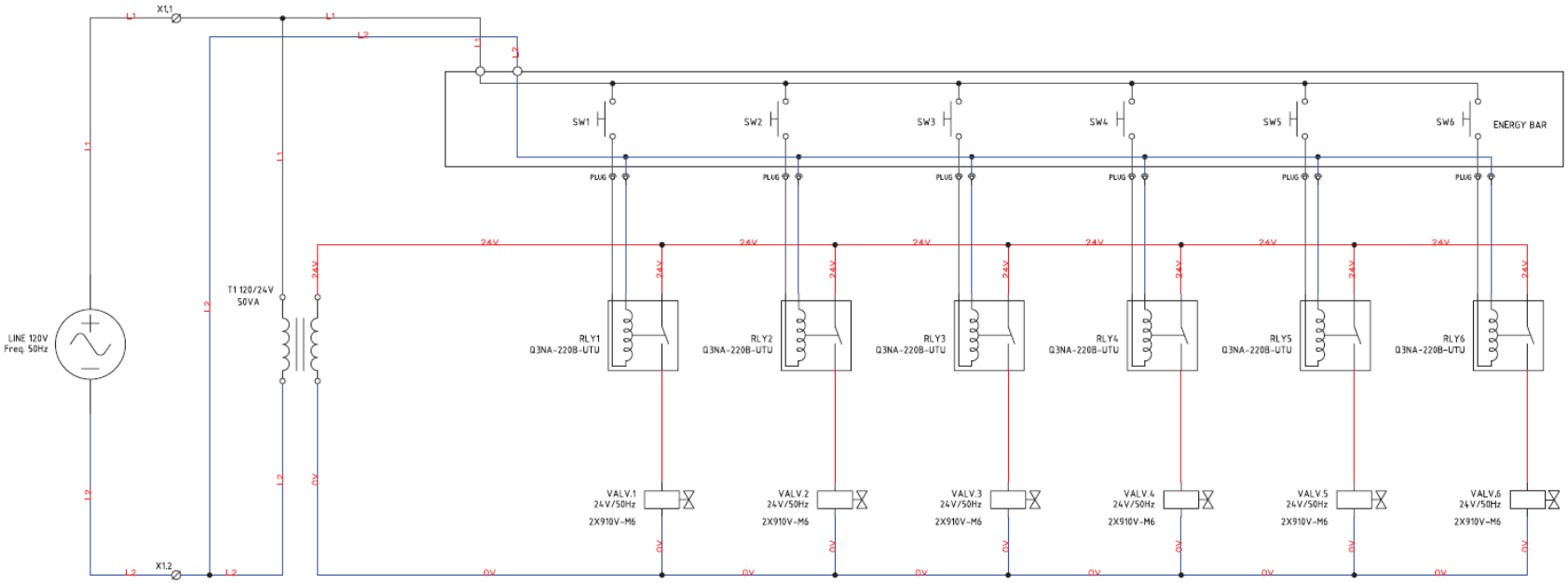

Figure 7. Electrical system designed to use the information collected from the Apex Fusion® to control the pH independently in each one of the bags in the bioreactor. The line (120V) is the voltage provided by the electricity company, T1 is the transformer, SW's are the switches in the energy bar (letter "b" on Figure 6), the RLY's are the solid state relays, and the VALV's are the valves in the manifold.

Figure 8. Interfaces of the Apex Fusion® app on the cellphone.

Figure 9. Cream milk electric centrifugal separator (version 100-18), Motor Sich JSC© used to separate the biomass and the liquid part from the bags containing dairy wastewater and water.

Figure 10. Chlorella sp. biomass collected inside the cream milk electric centrifugal separator (version 100-18), Motor Sich JSC©.

Chapter 3

The use of *Chlorella sp.* to remove nutrients from dairy wastewater to produce livestock feed

Synopsis

In this study, *Chlorella sp.* was cultured in mixotrophic conditions using different ratios of raw dairy wastewater in water, in bench-scale (1.25L) and pilot-scale (70L). The influence of extra CO_2, pH, temperature, solar radiation, photosynthetic active radiation, were tested for the cell growth, biomass productivity and nutrients (NH_4^+, PO_4^{3-}, NO_3^-) removal. The aim of this study was to find the best ratio (1:10, 1:20, 1:30 or 1:40) of dairy wastewater in water, where *Chlorella sp.* biomass could be produced, removing the highest amounts of nutrients possible, and then test the significance of adding extra CO_2 into the system. In the first experiment, lab and pilot-scale had the same biomass growth behavior, where control had the highest productivity and was followed by 1:10 (1.315 ± 0.240 g/L and 1.575 ± 0.599 g/L, respectively for lab-scale, and 0.752 ± 0.397 g/L and 0.434 ± 0.355 g/L, respectively for pilot-scale), both of them being harvested after seven days of experiment. Ratio 1:30 showed to be an alternative for reactions of four days long because 1:30 was not significantly different 1:10 when the goal was to remove nutrients. However, it was significant different for biomass production. In pilot-scale, none of the treatments (control, controlN, 1:10, 1:10N, 1:30 and 1:30N) were significantly different from each other, considering the nutrients (NH_4^+, PO_4^{3-}, NO_3^-) removal rates and biomass, when adding or not, extra CO_2.

3.1 Introduction

Microalgae represent an intricate and well-organized cell with multiple complex structures and mechanisms. Various studies indicate that microalgae are an ancestor of plants, moving from the ocean to the land their ability to transform the sunlight energy into chemical energy (Safi et al., 2014). However, it is still challenging to find the correct classification for microalgae since they are photosynthetic, meaning that they cannot be classified as Protists, and they are unicellular, meaning that they are not plants. Also, considering *Chlorella sp.* as the principal strain used in this study, with photosynthetic properties and single cells, they cannot be classified as bacteria (Larkum et al., 2003*).*

Adaptable to different ecosystems and types of interactions, microalgae need at least three key components to grow: sunlight, a carbon source and a moisturized environment (Rizwan et al., 2018). Responsible for about half of the world atmospheric oxygen production (Falkowski et al., 1998; Singhet al., 2011), microalgae naturally use carbon dioxide (CO_2) from the atmosphere as a carbon source, water (H_2O) as a source of electrons, oxygen (that will be released later) and sunlight (when working in a laboratory, sunlight is substituted for lamps) as the catalyst of the photosynthesis reaction. Moreover, it is possible because the microalgae have *chlorophyll α* – some species have *chlorophyll b* as well - in their cells, which makes it possible for the organism to transform the substrates (carbon dioxide and water) into carbohydrates and oxygen. Chlorophyll is an essential pigment for the photosynthesis to occur, used by the cell to transform the light into chemical energy. In microalgae, the most common type is *chlorophyll α,* which has a

maximum absorption range between 430*nm* and 450*nm*, and also between 673*nm* and 695*nm* (Barsanti & Gualtieri, 2014; Brown & French, 1959).

Able to adapt itself to various environments, microalgae have a vast niche. There are microalgae submerged in freshwater, hot springs (like the ones living in community with bacteria around the grand prismatic at Yellowstone, surviving the temperatures that can reach 70°C), living at the surface of water bodies (like at the Gulf of Mexico), and on dry surfaces, like rocks, soil and woods. The microalgae can also endure significant variations of pH, turbidity, oxygen and carbon dioxide (Barsanti & Gualtieri, 2014).

Algae blooms are being reported all around the world, caused mainly by the addition of fertilizers and animal manure on croplands (Chiu et al., 2015). It is causing severe eutrophication problems in all kinds and sizes of water bodies all around the world. Eutrophication is a process caused by excessive inorganic and organic nutrients – nitrogen and phosphorus, mainly - runoff into water bodies, affecting 65% of the estuaries and coastal waters in the U.S. that were already studied and analyzed by government agencies and universities (NOAA, 2017; Bengtsson et al., 2012). High levels of nutrients support the proliferation of various populations that can remove these nutrients, like bivalve mollusk and algae (Bennett et al., 2001; Tilman et al., 2001). The problem is that when algae blooms are involved in the process what is more likely to happen is a biofilm formation at the surface, impeding the sunlight to transpose it, causing the death of plants that are in the lake or ocean bed. The death of these plants will cause organic matter accumulation and oxygen (O_2) depreciation. The species that can move will probably scape this area, looking for survival. However, the stationary species will stay and die as the oxygen level decreases. Bacteria and other organisms that feed on the organic matter will

drive, increasing its population and releasing gases, like carbon dioxide (CO_2) and methane (CH_4). Depending on the size of the water body, and with a higher possibility of occurrence in lakes (because the water does not move a lot), it can come to deficient O_2 concentrations, killing most of the animals and plants underwater. In addition to that, the increase of CO_2 will decrease the pH, turning the water into a more acidic environment (Glibert et al., 2005; Rabalais et al., 2009; Moss, 2011).

Researched for many years, *Chlorella sp.* is a versatile microalgae species when used as a biological treatment for wastewater (Kebede-Westhead et al., 2006; Mulbry et al., 2008; Shi et al., 2016; Liu et al., 2017), being able to produce high content of lipids that could be converted in biofuels (Hu et al., 2012; Espinosa-Gonzalez et al., 2014; Lu et al., 2015; Church et al., 2017; Bindra & Kulshrestha, 2019) and/or high content of protein turning this microalgae species in a suitable option for human and livestock feeding (Yan et al., 2012; Kang et al., 2013; Szabo et al., 2013; Gruenwald 2014; Das et al., 2015).

It is crucial to have processes that are efficient and, if possible, combining steps to turn the processes more effective, spending less time and money. Even though microalgae are causing the depreciation of oxygen concentration in some water bodies, it is also a valuable resource when nutrients need to be captured and fixed (Singh et al., 2011). Because of the large scale dairy production, dairy wastewater is considered an environmental issue. Dairy wastewater usually is abundant in milk, fats, washing detergents (used to clean the facility) and nutrients (nitrogen – in the form of nitrates, ammonium and nitrite -, phosphate, sulfur and organic carbon), and because of nutrients content, most of the times, it is applied directly on croplands as a fertilizer (Cheung & Wong, 1981; Daneshvar et al., 2019). Therefore, combining microalgae properties on

removing nutrients from dairy wastewater and biomass growth, may result in environmental and economical solutions to help farmers improving water quality, by recycling nutrients, and producing a potential high protein product for livestock feeding. However, when thinking about applying this technology in farms, the high costs of adding extra CO_2 to enhance microalgae growth may hold producers back (Ramaraj et al., 2015; Liu et al., 2017). An evaluation of the difference in biomass production when adding extra CO_2 and when not, is needed, leading to an actuality implementation of the technology developed in this study.

This study is important and unique because was designed and conducted aiming to use a microalga strain, extracted from a dairy wastewater lagoon, to recycle the nutrients presented in that wastewater, while producing a high-value and rich *Chorella sp.* supplement to feed dairy cattle, all in and from the same place. It is important to note that this study closes a cycle, where the treated water can go back to the environment without been so harmful to it, while the nutrients kept by the microalgae can serve as feeding supplementation to cattle, with everything happening in the same farm.

The objectives of this study were: (1) conduct lab-scale experiments with different ratios of dairy wastewater in water aiming to recycle the highest amount of nutrient and to produce as much *Chlorella* biomass as possible; (2) conduct pilot-scale experiments (70L) with different ratios of dairy wastewater in water to optimize microalgae growth and nutrients removal rates outdoors; (3) Measure nutrients removal rates, growth capacity, and biomass yield during lab and pilot-scale experiments; (4) Evaluate the necessity of adding CO_2 as an extra source of carbon and pH regulator to optimize microalgae growth; (5)

Evaluate the biochemical composition of the biomass produced during the pilot-scale aiming to conduct palatable livestock feeding studies.

3.2 Materials and Methods

3.2.1 Algae cultivation

The *Chlorella sp.* used in this study was isolated from a dairy wastewater lagoon located at the West Central Research and Outreach Center (WCROC) in Morris, Minnesota – USA (45°35'38.0"N, 95°52'17.4"W), by researchers of the University of Minnesota. The dairy farm at the WCROC milk, approximately, two hundred cows, between conventional and organic, twice a day. The strain was chosen based on its ability to grow fast, to resist high pH, and to not be harmful to the livestock that would eat it. The microalgae were cultivated in BG-11 modified medium (based on Andersen, 2005, pages 435 and 436) which was called AM6 and is shown in Table 1. It was used to seed all the different experiments.

The medium used during lab-scale and pilot-scale experiments (dairy wastewater, AM6 and DI water) were not sterilized to see how the microalgae would respond to the toxicity and the interactions with other microorganisms. According to the experimental design, four different ratios would have a better response and balance between cell growth, biomass production and nutrients removal.

In order to find determine the better amount of DW where the microalgae could grow, produce more biomass and to remove the highest amount of nutrients, four different ratios of DW in water were tested, 1:10, 1:20, 1:30 and 1:40. Controls were carried out with AM6 medium. The different ratios experiment had an extra addition of CO_2 as an additional source of carbon and to control the pH. A second type of experiment was conducted to evaluate the necessity of adding extra CO_2 (to improve the chance of implementing this system in farms and to reduce costs). This second experiment was carried out using the ratios 1:10 and 1:30, and controls. The results of one system with extra CO_2 supplementation were compared to the other one without it.

3.2.2 Dairy wastewater

The dairy wastewater (DW) used in this study was pumped from the wastewater lagoon at WCROC and reserved in a funnel-shaped tank to settle by gravity between three and five days prior the first use, either for lab or pilot-scale. The DW (Table 2) used during the experiments was taken from the top of the tank and used in reactors in different percentages diluted in water. The water used during lab-scale experiments was deionized water (DI) and tap water (with hard water properties) for pilot-scale.

3.2.3 Bioreactors design

3.2.3.1 Lab-scale bioreactors

It is composed of two boxes made of glass (height = 0.48m, length = 0.76m, width = 0.31m) opened on top, with an acrylic support for eight glass tubes (diameter = 0.075m, height = 0.05m) positioned in parallel to each other. Rubber stoppers were used to keep the system closed. A glass tube (diameter = 3mm) passing through the rubber stopper was used to inject air into the system and CO_2 when needed. Each one of the systems had one blower injecting air at a rate of 0.8 L/min ± 0.1 L/min in each one of the tubes, adjusted by flowmeters. CO_2 was used to control the pH and as a carbon source in some of the experiments.

The CO_2 was added without being diluted, but it was mixed with air before going into the glass tube and inside the reactor. The pressure was 17kPa (16psi) in the way out of the cylinder. The lights used during the lab-scale experiment were bought from Philips, model F28T5/830 ALTO, warm white, 2900 lumens, and 28 watts. The lab-scale experiments took place at the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) – Soils Management Research, at Morris, Minnesota.

3.2.3.2 Pilot-scale photobioreactors

The pilot-scale photobioreactors (PBR) were designed to have six bags in one system, where all of them could receive approximately the same amount of solar radiation during the day. The structure was made using treated pinewood (height = 1.73m, length =

2.06m and width = 1.22m). Three pars of flexible metal bars (diameter = 0.05m, height = 1.34m), distant 0.03m from each other, were installed to support the shape of the hanging bags. PVC tubes (diameter = 0.015m, height = 1.33m, length = 0.52m) were used to blow oxygen into the bags and carbon dioxide, when needed. At the bottom of each PVC tube, twelve holes (diameter = 0.5mm) were drilled for the air to flow, aerating and mixing the whole system.

The PBR's (pallet 1 and pallet 2) were set up outside of the dairy barn at the West Central Research and Outreach Center (WCROC) in Morris, Minnesota. Each one of the pallets had a box attached in one of the sides, containing a system to monitor pH and temperature, and control the pH. A liquid CO_2 tank was used as a carbon source and to control the pH when needed. Each one of the bioreactors (two total) had a capacity for six hanging bags with a maximum volume of 80L each, but all experiments were conducted in 70L.

The airflow was measured using an 8360 VelociCalc® Plus from TSI, and it was flowing in a rate of 4.61 ± 0.09 m^3/min from the blower into the bag through the larger black hose (this rate was measured in each one of the bags individually). The air flowing system was composed of a norprene hose (diameter = 0.48cm) to add carbon dioxide, and a rubber hose (diameter = 3.5cm) to insert air from the blower into the bag. To not insert 100% CO_2 (CO_2 was added only during specific experiments) into the system, ideally the two fluids were mixing while entering together with the PVC tube and then flowing into the algal system.

3.2.4 Temperature and pH monitoring and controlling system

The Apex Fusion® consists of an electronic system created by the Neptune company (from California) to monitor different parameters in aquariums. During this study, the Apex Fusion® was used as part of a temperature and pH monitor system, and a pH controller system. The items bought from Neptune are: an energy bar, probes' modules, and an Apex controller. The main objective of using this system was to use the pH probes from the Apex Fusion® system to determine what was the pH and the temperature in each one of the bags. Using the pH information, it was possible to program the Apex Fusion® to turn on a solid states relay, opening a valve from a manifold and releasing CO_2 automatically in each one of the bags when the pH reached a maximum value (8.6) and then closed the valve when the pH reached the minimum value (7.4). The pH values could go further down than 7.4 because there was a delay between the measurement and closing the valve.

In summary, what the monitoring and controlling system does is: the temperature and pH probes collect the measurements, and they stay saved in the Apex Fusion® controller, when the pH probe has a measurement over 8.6 it will inform the Apex Fusion® controller to unlock one the designated switch and turn on the solid state relay (RLY) for the specific bag. The RLY will open one of the manifold valves, and the valve will release CO_2 inside the bag until the pH probe gets a measurement below 7.4, then the Apex controller will lock the switch, turning off the RLY and the valve. To monitor the temperature, a temperature probe was positioned inside a random glass tube for each one

of the aquariums during the lab-scale experiments, and for the pilot-scale, each one of the bags had its individual temperature probe.

During lab-scale the temperature inside the glass tubes varied between 22.8°C and 29.1°C. In the pilot-scale experiments, the temperature variation was between 18.2°C and 40°C.

3.2.5 Light monitoring: PAR and solar radiation

During the lab-scale, in addition to control and monitor the pH, and monitor the temperature, it was possible to use the Apex Fusion® system to monitor the photosynthetic active radiation (PAR) that the microalgae culture was receiving from the lights. The PAR sensor was positioned in between tubes, to measure the photosynthetic active radiation that the tubes were receiving and it varied between 331 and 338 $\mu mol/m^2/s$. Furthermore, the lights were set up to be on every day between 5 a.m. and 9 p.m. to imitate the duration of the sunlight received by the bags during pilot-scale.

Through pilot-scale experiments, the bioreactors were positioned outside, exposed to ambient changes between July and August 2018. The orientation of the bags was east to west. The solar radiation during pilot-scale was measured using the data from the United States Department of Agriculture – Agricultural Research Service (USDA-ARS), that have permanent experiments going on near Swam Lake, which is approximately 11km from the WCROC, where the PBR's used for the wastewater treatment were located. The information provided by the USDA-ARS was an average of the radiation for the day and

ranged between 40.56 and 303.55 W/m^2. To be able to compare lab-scale and pilot-scale, an approximate conversion was used, where, for sunlight conversion, 1 µmol/m^2/s was equal to 4.57 W/m^2 (Langhans, R.W., et al., 1997), it means that the solar radiation received by the pilot-scale PBR's varied between 185.34 and 1387.23 µmol/m^2/s.

3.2.6 Cells count and dry weight biomass estimation

The hemocytometer protocol (Absher, 1973), using a Neubauer chamber, was used to determine the cell density (cells/L). The cells density was determined by Equation 1, where SC is the average cells per small square, D is the dilution factor, and V is the volume of a small square in mL (in this case, $V = 0.0001mL$).

$$Cell\ density = \frac{SC*D}{V} \quad \text{(Eq. 1)}$$

For the biomass estimation, a 0.22µm nitrocellulose membrane was left to for at least one hour, weighted, then a sample of 15mL was filtered through it using a vacuum pump. The filter was then put back to dry at 65°C until the weight was constant. The difference in the membrane weight estimated the dry weight biomass (g/L) on that day for each one of the treatments.

3.2.7 Nitrate, Ammonium and Phosphate concentrations

In order to determine the daily concentration of nitrate, ammonium and phosphate, 30mL of the sample was filtered using a 0.22µm nitrocellulose membrane (Merck Millipore Ltd.) in a 500 mL filtration unit (Nalgene), and a vacuum pump. The same sample was used to determine the concentrations of nitrate and ammonium (15mL). It was stored in a refrigerator at 8°C after 0.3 mL of concentrated sulfuric acid was added to the sample. The phosphate sample (15mL) was stored at -4°C after being filtered. These samples were analyzed using Lanchat.

3.2.8 Harvesting techniques

The lab-scale experiments were not harvested because of the small amount of biomass produced. In that case, for the lab-scale, only the biomass production per day was evaluated filtering 15ml of the sample through a 0.22µm filter paper. However, for the pilot-scale, two different harvesting techniques had to be used.

3.2.8.1 Harvesting *Chlorella sp.* from artificial medium in pilot-scale

When harvesting the biomass from the pilot-scale experiments, the blower was turned off after the last sampling. The microalgae biomass was expected to precipitate after

three to five hours, accumulating in the bottom of the 70L bag. However, what was observed for the controls, was that the microalgae would stay distributed in the entire water column, having only a minimal amount going to the bottom. Because of that, the entire bag had to be centrifuged. In order to make the process more efficient and less time consuming, a cream milk electric centrifugal separator (version 100-18), designed by Motor Sich JSC©, was used. The initial capacity of this product is to separate 100 L in one hour, with 75 rpm. However, to separate microalgae, some modifications had to be made, including not using the twelve disks that are supposed to be inside the centrifuge. For this project, the microalgae would stay trapped inside the centrifuge, and the liquid part would leave the separator system. Because of that, every time that the water leaving the system started to be dark green, the separator was turned off, and the biomass was harvested from inside the centrifuge system. After collecting the biomass, it was sterilized under 121°C and a pressure of 15 psi, for 30 minutes. The biomass was then kept frozen at -4°C after drying in an incubator for eight days at 65°C.

3.2.8.2 Harvesting *Chlorella sp.* from different ratios of dairy wastewater in water in pilot-scale

After taking the last sample of each experiment, the blower was turned off, making it possible for the microalgae biomass in the bags containing dairy wastewater, to precipitate after a minimum of 3 hours. In this case, less than five liters of the mixture of water and wastewater containing *Chlorella sp.* biomass had to be centrifuged using a Sorvall Legend XTR Centrifuge, from Thermo Scientific, at 6500*xg* during 2 min. The

biomass was collected, sterilized under 121°C and a pressure of 15 psi, during 30 minutes. The biomass was then kept frozen at -4°C after drying in an incubator for eight days at 65°C.

3.2.9 Statistical Analysis

3.2.9.1 Data analysis of the different ratios of DW in water for lab and pilot-scale

The ratios experiment during lab-scale was one week long (two replicates total) with duplicates of five different ratios (control, 1:10, 1:20, 1:30, and 1:40). The same experiment was conducted in pilot-scale (three replicates, one week each) with duplicates of five different ratios (two controls, 1:10, 1:20, 1:30, and 1:40). During both scales, data was collected from day 0 through 6 for the ratios 1:30 and 1:40, and from day 0 to day 7 for control, 1:10, and 1:20. All the ratios in this experiment were supplemented with an extra addition of CO_2.

Day 0 to 6 data were analyzed using a mixed model in SAS (SAS Institute Inc.) with fixed effects of day, ratio, and the day and ratio interaction. Covariates of temperature, pH, and solar radiation were included if F-test was meaningful ($P < 0.05$). Random effects were replicate, bag, and replicate and pallet interaction. The repeated effect was day specified by the first-order autoregressive covariance structure. The P-values were adjusted using the Tukey procedure. A different model was built to analyze day 7 for the ratios 1:10, 1:20, and control. However, it was analyzed with the same technique as day 0 through 6. The data were log transformed and back transformed to be reported.

3.2.9.2 Data analysis of the different ratios of DW in water for lab and pilot-scale when investigating the necessity of CO_2 supplementation

When the addition of CO_2 was investigated, the experiments in lab-scale were one week long (two replicates) with duplicates of six different ratios (control, 1:10, 1:30, controlN[1], 1:10N, and 1:30N). Throughout pilot-scale, an one week experiment was run with duplicates of each one of the six ratios (control, 1:10, 1:30, controlN, 1:10N, and 1:30N). Either in lab or pilot-scale, data for the control, controlN, 1:10, and 1:10N was collected from day 0 to day 6. Although, for ratios 1:30 and 1:30N, data was collect between day 0 and day 4.

Using SAS (SAS Institute Inc.), day 0 to 4 data were analyzed using a mixed model with fixed effects of day, ratio, and the day and ratio interaction for all the different ratios. Covariates of temperature, pH, and solar radiation were included if F-test was meaningful ($P < 0.05$). Random effects were replicate, bag, and replicate and pallet interaction. The repeated effect was day specified by the first-order autoregressive covariance structure. P-values were adjusted using the Tukey procedure. A different model was built to analyze days 5 and 6 for the ratios control, controlN, 1:10, and 1:10N. However, the same model used for day 0 through 4 was to analyze the data for these last two experimental days. The data were log transformed and back transformed to be reported.

[1] N means that the designated tube or bag did not receive CO_2 supplementation during the experiment.

3.3 Results and Discussion

3.3.1 Finding a ratio of dairy wastewater in water to optimize nutrients removal and microalgae biomass production

3.3.1.1 Lab-scale

During lab-scale, cell density was observed to be higher in the control (Figure 1), followed by ratios 1:10 and 1:20, respectively (Table 3). Temperature and day were significant effects ($P < 0.05$) on the cell density during this experiment. However, in the first six days of the experiment, only the control and ratio 1:40 were significantly different, while on day 7 control and 1:20 were significantly different. Temperature and dilution were not significant ($P > 0.05$) effects for the ratios harvested on day 7. Delgadillo-Mirquez et al. (2016) also ran experiments in lab-scale (200 mL) with medium where the microalgae was mixed with bacteria cultures, and the temperature was a significant effect on cell growth, as found in the present study. However, the authors also found that the temperature influenced the biomass productivity, phosphate removal, and ammonium removal, what was not found in the models of this study in lab-scale.

The biomass for lab-scale had a considerable variation between experiments on day 7 (Figure 2). On the harvesting day, control had the highest cumulative production, followed by the ratio 1:10 and 1:20 (Table 4). During lab-scale, the only effects influencing the biomass productivity were day, ratio and the interaction between day and ratio. Before

harvesting the ratios 1:30 and 1:40, on day 6, the only significant different comparison was between 1:40 and the control. On day 7, when the other ratios and the control were harvested, there was no significant difference between the control and the 1:10, which suggests that 1:10 is a promising ratio to produce biomass to carry feeding studies with ruminants, or to produce biofertilizer while removing nutrients from wastewater. Åkerström et al. (2014), cultivated *Chlorella sp.* using different dilutions of sludge liquor in municipal wastewater, and the lowest concentration was 12%, very close to the ratio 1:10 used in this study. The authors harvested 0.96 ± 0.18 g/L of dry weight biomass after ten days of treatment, which is close to the dry weight produced during this study for the ratio 1:10 (1.575 ± 0.599 g/L).

The daily phosphate removal density are presented in Figure 3 and Table 5. Except for day 0, the controls were significantly different from all the ratios in the experiment. The ratios were not different from each other on any experimental day. On day 6, control and 1:10 were significantly different from all the other treatments. For the ratios and the control harvested on day 7, no difference was observed in the phosphate removal density. Considering that, the lowest ratio between DW and water, 1:10, is also promising to remove considerable amounts of phosphate from dairy wastewater, 73.13% in this study.

Figure 4 is presenting the removal density of NH_4^+ in lab-scale, where 1:10 was significantly different from ratios 1:30 and 1:40, on day 0. The ratios 1:30 and 1:40 were harvested on day 6 for this experiment, and there was no difference in the ammonium levels between any of the other ratios or the control. However, treatments harvested on day 7 were all different from each other. Throughout this experiment, control removed 99.21%

of the NH_4^+ , while the ratios 1:10 and 1:20 removed 99.53% and 99.37%, respectively (Table 6).

Figure 5 shows the nitrate removal density for the lab-scale experiment. 1:30 and 1:40 were harvested on day 6, where all the tubes showed no difference on the nitrate concentrations, and the same was observed for the tubes harvested on day 7 (Table 7). Temperature, day (0 - 6), ratio, and the interaction between day and ratio were significant variables for the nitrate removal. No significant difference was observed for any of the effects on the tubes harvested on day 7. The highest nitrate removal density were found for the control (97.03%), 1:10 (74.96%), and 1:20 (36.35%), at the last day of the experiment.

3.3.1.2 Pilot-scale

Cell density during pilot-scale was observed to be higher in the control (Figure 6), followed by ratios 1:10 and 1:20. Day 0 was not significant ($P > 0.05$) different in any of the treatments regarding cell density (Table 8), meaning that the bags had a similar number of cells/L at each of the three different batches in this experiment. Day (0 - 6), ratio, and the interaction between day and ratio had a significant effect on cell density. In this case, cell density is dependent on the temperature and solar radiation. For the ratios that were harvested on day 7, temperature and solar radiation were not significant and the only variable significant for the model was ratio.

When running the experiment in pilot-scale and trying to find the best ratio between DW and water, again the control bags with AM6 artificial medium were performing better than the bags with DW in water (Table 9). There was no difference in the initial biomass weight between bags on day 0. However, all the bags had different biomass yield on the last experimental day when comparing to the control (Figure 7). Control produced an estimate of 0.64g/L (0.39 -1.05g/L) of biomass, while 1:10 (DW in water) produced 0.33g/L (0.22-0.50 g/L) in the last day. Day (0-6), ratio, and the interaction between day and ratio were significant variables for biomass productivity. In this case, biomass productivity is dependent on the pH and solar radiation. For the ratios that were harvested on Day 7, pH and solar radiation were not significant, and the only variable significant for the model was ratio.

Daily phosphate (PO_4^{3-}) removal density are presented in Figure 8 and Table 10. The controls were significantly different from all the ratios every day of the experiment because the AM6 medium contains a high amount of prosperous in its recipe. The ratios were not significantly different from each other on any day. Coincidentally with the last day of the log phase for cell growth on ratios 1:10, 1:30, and 1:40, the day with less amount of phosphate dissolved in the DW/water was day 4. After day 4, the cells started to deplete in number, and the quantity of phosphate started to go up again. It is important to emphasize that the DW was used without being sterilized, meaning that all the bacteria and pathogens that are found in this type of effluent were not killed before the experiment started. These organisms may influence the fluctuation of phosphate concentration. Day (0 - 6), ratio, and the interaction between day and ratio had an effect on PO_4^{3-} removal. In this case, phosphate removal is dependent on temperature. For the ratios that were harvested on day

7, temperature was not significant and the only variable significant for the model was ratio. Whitton et al. (2016) reported a low phosphate removal density, ranging from 12.5 to 19.6%, while in this study, 1:10 removed 27.05% and 1:30 21.54%. Also, the authors present results where *Chlorella vulgaris* had an increase in the phosphorous concentration after ten days of remediation trials. Moreover, comparing the cell growth (Figure 6) to the phosphate removal (Figure 8), the cells start their stationary phase between the third and fourth day, exactly when the phosphate concentration goes down and supporting the phosphate as one of the main nutrients that algae need to grow and regulate their metabolism (Powell et al., 2008; Kim et al., 2012; Razzak, et al., 2013; Ramaraj et al., 2015).

Figure 9 shows the removal density of Ammonium (NH_4^+), where the controls were different from all the DW/water ratios every day of the experiment. However, the different ratios were not different from each other on any day. All the ratios and control harvested had very high removal density, dropping the levels close to zero in all three different weeks. Control removed 98.12%, while the ratios 1:10 and 1:30 removed 97.55% and 95.53%, respectively (Table 11). Day (0 - 6) and ratio were significant variables for Ammonium removal. In this case, NH_4^+ removal is dependent on solar radiation. For the ratios harvested on day 7, solar radiation was not significant, and ratio was the only variable significant for the model. Whitton et al. (2016) performed similar experiments to the ones presented in this study, where they had batch reactions for 10 days using dilutions of wastewater in artificial medium (BG11) and a mix of different microalgae, including *Chlorella*. They found ammonium removal density above 99% in liquid phase reactions.

Nitrate removal density are shown in Figure 10 and Table 12. Control was lower during the length of the study, but none of the ratios were significantly different from each other on any day. All bags, except for the ratio 1:30, had the same behavior with respect to nitrate removal density. A decrease was observed after day 3 in the bags mentioned above (when the cells were still in log phase - Figure 6). The highest removal density were observed for the control (84.11%), 1:10 (39.27%), and 1:20 (34.96%), on the last day of the experiment. Day (0 - 6), ratio, and the interaction between day and ratio had an effect on nitrate removal density. For the ratios harvested on day 7, ratio was the only variable significant for the model. Considering the balance between N:P in this reaction, it is important to compare Figures 8, 9, and 10, where a decrease in the phosphate concentration occurs in the first three days, when the levels of ammonium and nitrate maintain close to the initial amount, being used only after the fourth day and going close to zero in most of the ratios, including the control. These fluctuations were noticed by many authors that have tested different N:P ratios and its variation in different experimental time (Beuckels et al., 2015; Choi & Lee, 2015; Åkerström et al., 2016).

3.1.1 Testing the necessity of extra addition of CO_2 as a carbon source and to control pH when using different ratios of dairy wastewater in water

3.1.1.1 Lab-scale

Due to the high costs of adding extra CO_2 to systems that may be applied in farms, where people can clean the wastewater and produce livestock feed *in-situ*, this experiment aimed to test the ability of the *Chlorella sp.* to grow and remove nutrients from DW, without adding extra CO_2 when running the reactions using PBR's.

Regarding the production of microalgae biomass during lab-scale temperature was the only significant effect ($P < 0.05$) between days (0-4). Moreover, on day 4, when the ration 1:30 was harvested, there was no significant difference in the biomass productivity between the different dilutions (Table 13 and Figure 11). Furthermore, on days 5 and 6, the temperature was not significant for the model anymore, and CO_2 continued not to make a difference in biomass productivity. In lab-scale the proportion of biomass production in the treatments with extra CO_2 is higher when placing the three treatments together (Figure 12), being significant different in the last two days of experiment. However, between day 0 and 4 no significant difference was observed in the biomass productivity (Table 14).

The higher phosphate removal density was observed in the ratio 1:30 for both experiments, and when extra CO_2 was added (removal density as high as 96.11%) and when it was not (removal density as high as 98.61%). Due to the up and downs that make it hard to interpret the results (Table 15), the phosphate data were not analyzed as the other data were. For example, the results for the controls', that started with a low level and for both experiments they went up and reached the higher pick on day 3. After that, the levels went down again but went back up on the last day of the experiment. The same trend is not noticed in the other ratios that had a high value on day 0 and continue to go down in the following days. When considering only the extra addition or not of CO_2, the tubes that

did not receive carbon dioxide had a significant lower concentration of the gas than the tubes with extra amounts of CO_2.

For the first four days of the experiment, the nitrate concentration were significantly different between the control and the ratios 1:10, and 1:30, but it was not different between the two ratios (Table 16). Furthermore, during the last two days of experiments, no significant difference was found in the results between the control and 1:10. Temperature, ratio, and the interactions between day and CO_2, and day and ratio were significant effects in the nitrate removal density, from day 0-4 (Figure 13). Figure 14 shows the results regarding the extra addition of CO_2, where no difference was found in the first four days for the nitrate removal density. However, for day 5 and 6, all the effects mentioned before were not significant, but CO_2 was (p-value = 0.0421). Even though the tubes with extra CO_2 seemed to have higher nitrate removal density in the final day, but the tubes without extra gas removed higher rate throughout the experiment, 78.66% without extra CO_2 and 75.18% with extra CO_2 (Table 17).

Ammonium concentration from day 0 to 4 were significantly different only for the ratio 1:10 and 1:10N. For the last two days of experiments, there was no significant difference between the dilutions (Table 18 and Figure 15). Furthermore, during the first four days of the experiment, temperature, the different ratios, and the interaction between the ration and addition of CO_2 were significant effects on the model. However, none of the effects listed above were significant during days 5 and 6 of this experiment. Figure 16 and Table 19 show the data regarding the influence of the extra addition of CO_2 in the ammonium concentration, and there was no significant difference in none of the experimental days. On the last experimental day, the dilutions without extra CO_2 removed

up to 96.56% of the initial ammonium concentration, while the tubes with extra CO_2 addition removed up to 97.74%. Wang et al. (2010), seemed to not have added CO_2 during their nine-day experiments involving four different wastewater from the same treatment plan, and the authors could remove 74.7% of the NH_4^+ from the wastewater used in this experiment, 90.6% of the PO_4^{3-}, and up to 62.5% of the NO_3^-. Liu et al. (2017), found that *Chlorella vulgaris* take long period of time to deplete ammonium from wastewater when no extra CO_2 is added to the system. For example, they found that for the microalgae to deplete NH_4^+ close to zero, would take three days for concentrations of 1%, 5%, and 10% if CO_2, three and a half days for 20% of CO_2, and five days and a half when not mixing CO_2 in the aeration system. This result supports the findings for ammonium concentration of the study presented in this thesis.

3.1.1.2 Pilot-scale

Cell density had the higher concentration in the control and ratio 1:10 that had the extra CO_2 (Figure 17 and Table 20). Day and the interaction between day and ration were the only significant effects for this model during day 0-4. Although, they were not significant ($P > 0.05$) on the last two days of the experiment when the CO_2 was the only significant effect influencing in the cells' density. *Chlorella sp.* is known as a species that has its growth limited when CO_2 levels exceed 10% (v/v) (Silva & Pirt, 1984; Lee & Tay, 1991, Cheng et al., 2006). However, Klinthong et al. (2015) stated that some *Chlorella sp.* can grow without added CO_2, in levels of up to 15%. Moreover, some species of that microalgae can grow with the addition of CO_2 in concentrations of up to 100% and tolerate

it, supporting the findings of the study presented in this thesis, where *Chlorella sp.* biomass can be produced by mixing 100% CO_2 in different treatments, including the ones using DW in PBR's.

Regarding the biomass productivity (Figure 18), the higher amounts were produced by the controls and followed by the ratios 1:10. The data (Table 21) analysis showed no significant difference between the ratios on day 0 through 4, but the day and the interaction between Day and ratio were significant effects in this model. For days 5 and 6, the only significant effect was the different ratios. Therefore, CO_2 did not affect biomass productivity during this experiment (Table 22 and Figure 19). However, what was noticed during this entire experiment, was the formation of biofilm in the bag's walls, which did not allow the proper sampling for cells and biomass findings. Because of that, when the harvest occurred for each one of the bags, the higher biomass weight was harvested from the control with the extra addition of CO_2, and the amount collected was significantly different from the ratios 1:10 and 1:30 in the same conditions. However, after harvesting the bags where the extra CO_2 was not added, there was no significant difference between the controlN and 1:10N, but both of them were different from the ratio 1:30N (Table 23).

Figure 20 is showing the trends for phosphate removal in the different ratios when CO_2 was and was not added. Also, the results on Table 24 show that all the ratios that had extra addition of carbon dioxide removed higher density of phosphate than the ratios without extra addition. The maximum removal density were registered on day 4 for all the bags, having 1:30 as the ratio with higher removal density, either with extra CO_2 (98.29%) or not (93.05%), followed by 1:10 (82.80% with extra CO_2 and 72.11% without extra amount of the gas), and the controls (45.68% with extra CO_2 and 15.79% without additional

amount of the gas). From day 0 to 4 of the experiment, instead, the day, ratio, and the interaction between day and extra CO_2 were. The same was noticed for the model used for days 5 and 6, but the only significant effect was the different ratios. Moreover, the same tendency was found during the experiment to find the best ratios to carry this study, shown in Figure 8. The extra addition of CO_2 was not a significant effect in any day of the experiment, as shown in Figure 21 (Table 25).

For this experiment, nitrate removal density showed to be dependent on the day, ratio, and the interaction between day and ratio, and on days 0 and 4. Although, the nitrate removal was dependent on the ratio only, for days 5 and 6. Day 4 was the day that the ratios 1:10's and 1:30's, had the maximum removal density during the experiment. The controls removed the maximum nitrate amounts on the last day of the experiment (Figure 22). During this experiment, the ratio 1:10N had a removal density 69.78% in the last day, and the control with the extra addition of CO_2 removed 73.66% on day 6 (Table 26). Furthermore, there was no significant difference between the ratios and the extra addition of CO_2 in any day of this experiment regarding the nitrate removal density, but overall the ratios and the control with the additional amount of the gas removed higher density of nitrate (Figure 23 and Table 27).

Initially, the amount of ammonium was higher in the controls comparing to the ratios because of the type of medium used (AM6) during the experiments. However, at the harvesting days, all the bags had a similar concentration of ammonium (Figure 24) where the estimates were below 1ppm. The control without extra CO_2 had the highest ammonium removal density, 94.47%, followed by the ratio 1:10, also without extra CO_2, which removed 92.19% throughout the experiment (Table 28). The bags with an extra addition of

CO_2 showed to overall remove less ammonium (Figure 25 and Table 28), but adding extra CO_2 showed to not be a significant effect in any of the experimental days for ammonium removal.

3.1.2 Biomass biochemical characteristics

Table 30 presents the biochemical composition of the biomass produced during this entire, what was found to have high protein content (49.2%), low fat (2.32%), no mycotoxins, and good concentration of various minerals, such as calcium, iron, and zinc. This composition gives to the biomass produced during this study a high chance to be used as livestock feed, if they accept to eat this product. In animal feeding, microalgae can provide essential nutrients, minerals, vitamins, fatty acids and a high protein content, which is difficult to find all in the same organism (Madeira, M. S. et al., 2017; Kotrbacek, V. et al., 2015; Christaki, E. et al., 2011).

3.4 Conclusions

The lab-scale study showed promising results of biomass production and nutrient removal, being able to grow considerable amount of biomass in the different treatments (control: 1.315 ± 0.240 g/L; 1:10: 1.575 ± 0.599 g/L; 1:20: 1.005 ± 0.301 g/L; 1:30: 0.985 ± 0.370 g/L; 1:40: 0.800 ± 0.329 g/L) and to remove expressive nutrients levels, such as 73.13% of the phosphate, 99.53% of the ammonium, and 74.96% of the nitrate in the ratio

1:10. Considering the results, the same experiment was set up in pilot-scale (70L for each treatment) having control (0.752 ± 0.397 g/L) and the 1:10 (0.434 ± 0.355 g/L) as the best biomass producers, while control removed 98.12% and the 1:10 removed 97.55% of the ammonium, the control removed 84.11% of the nitrate and 1:10 removed 39.27%. Also, the controls could remove 9.85% of the phosphate, when 1:10 removed 27.05%. Being the lowest ratio between DW and water, 1:10 was the most efficient treatment in this study, using more dairy wastewater, producing the highest amount of biomass between the different DW/water ratios, and removing high density of phosphate, ammonium, and nitrate. Ratio 1:30 showed to be an alternative for short periods of time where the goal would be removing nutrients (not significant different from any of the removal density of 1:10 in this study) but not producing biomass.

The PBR designed for this study was meant to be used on farms, where the producers could recycle the wastewater and produce enriched biomass to use in their properties. To be able to do that, an experiment comparing three different treatments (control, 1:10, and 1:30) with and without the addition of extra CO_2 was carried out. The treatments 1:30 and 1:30N were harvested on the fourth day, where they could remove a high density of nutrients, but the biomass productivity was very low. Control, controlN, 1:10, and 1:10N were harvested on the sixth day, producing higher amounts of biomass than the treatments 1:30 and 1:30N. However, in pilot-scale, none of the treatments were significantly different from each other, considering the nutrients (NH_4^+, PO_4^{3-}, NO_3^-) removal density, or biomass, when adding or not, extra CO_2.

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Table 1. AM6 medium.

Component	Concentration (g/L)	Molar Mass (g/mol)	Concentration in Final Medium (M)[1]
$NaNO_3$	0.25	84.99	2.94×10^{-3}
NH_4Cl	0.05	53.49	9.34×10^{-4}
$MgSO_4.7H_2O$	0.075	246.47	3.04×10^{-4}
$CaCl_2.2H_2O$	0.025	147.01	1.70×10^{-4}
NaCl	0.025	58.44	4.28×10^{-4}
$(NH_4)_5[Fe(C_6H_4O_7)_2]$	0.01	261.98	3.82×10^{-5}
K_2HPO_4	0.025	174.20	1.44×10^{-4}
Na_2CO_3	0.025	105.99	2.36×10^{-4}
Trace elements solution	1mL/L	-	-

Trace Elements Solution	
Component	Quantity used (g/L)
H_3BO_3	0.6
$MnCl_2.4H_2O$	0.25
$ZnCl_2$	0.02
$CuCl_2$	0.015
$Na_2MoO_4.2H_2O$	0.015
$CoCl_2.6H_2O$	0.015
$NiCl_2.6H_2O$	0.01
V_2O_5	0.002
KBr	0.01

[1] Molarity = Concentration / Molar Mass

Table 2. Raw dairy wastewater properties.

Parameters	Concentration (mg/L)	Method
Biochemical oxygen demand (BOD)	498	SM 5210 B-(2011)
Chemical oxygen demand (COD)	3552	SM 5220 B-(2011)
Ammoniacal Nitrogen	301	SM 4500 NH3 C-(1997)
Nitrate/Nitrite Nitrogen	0	EPA 353.2
Carbon (total)	1630	SM 5310 B-(2011)
Total Phosphorus		

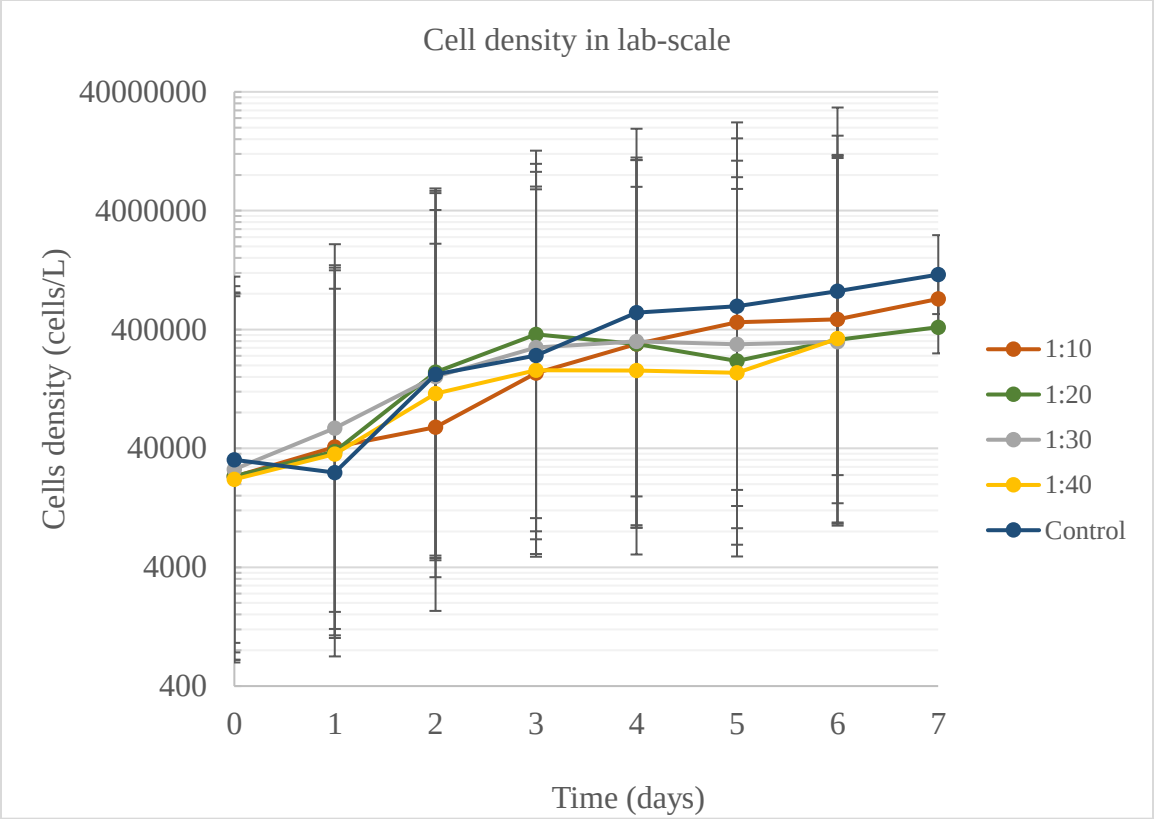

Figure 1. Cell density in lab-scale during the experiment to find the best ratio of DW in water.

Table 3. Least square means for cell density in lab-scale during the experiment to find the best ratio of DW in water (n = 20).

	Estimate				
	Ratio				
Day	1:10	1:20	1:30	1:40	Control
0	23161.1	23344.8	26684.3	21954.6	32087.8
1	41016.5	37662.9	59309.8	35765.2	25030.0
2	60158.0	175681.8	160448.7	115581.3	167666.1
3	172203.1	364043.9	282047.2	181612.4	241573.7
4	302952.0	302830.9	318134.6	180327.5	555558.8
5	461312.0	217668.8	299607.9	173343.4	629216.0
6	486275.1	327102.8	315284.2	335014.7	838801.9
7	723625.9	418540.9			1159535.2

	Standard Error									
	Lower					Upper				
Day	1:10	1:20	1:30	1:40	Control	1:10	1:20	1:30	1:40	Control
0	22494.9	22680.2	25916.0	21322.5	31164.0	782025.9	796794.8	900242.5	740676.6	1082538.6
1	39806.7	36593.7	57626.1	34749.9	24319.5	1349561.2	1289202.5	2029969.7	1224118.3	856690.0
2	58441.9	170669.6	155871.6	112283.8	162883.1	2048431.5	5982732.0	5463416.9	3936048.6	5709174.2
3	167300.9	353687.8	274023.7	176446.5	234702.2	5877560.9	12434344.5	9633651.7	6202543.4	8251230.7
4	294346.8	294234.2	309103.5	175207.9	539787.8	10362601.5	10364856.0	10887528.2	6171987.2	19014861.4
5	448211.2	211481.0	291089.9	168415.7	611329.0	15782617.3	7438557.9	10239777.8	5924403.0	21504880.7
6	472457.1	317809.7	306326.9	325496.8	814971.2	16628108.5	11186385.6	10781099.4	11456960.7	28685663.5
7	290393.4	166554.6			619694.3	484921.6	276641.7			1331053.8

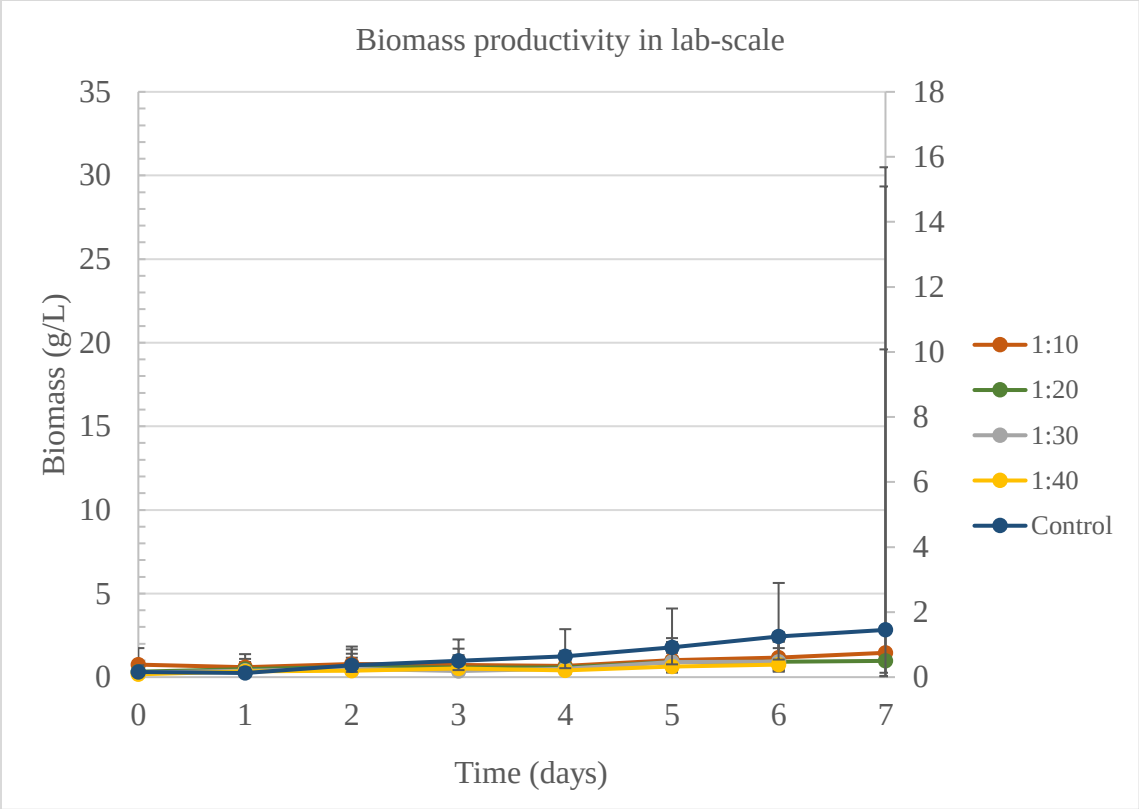

Figure 2. Biomass productivity per day of experiment on lab-scale during the experiment to find the best ratio of DW in water. Primary vertical axis (on the left) is correspondent to the results for ratios 1:10, 1:20, 1:30, and 1:40. Secondary vertical axis (on the right) is correspondent to the results for the control.

Table 4. Least square means for biomass productivity in lab-scale during the experiment to find the best ratio of DW in water (n = 20).

	Estimate				
	Ratio				
Day	1:10	1:20	1:30	1:40	Control
0	0.750	0.327	0.272	0.167	0.159
1	0.594	0.471	0.391	0.338	0.127
2	0.790	0.605	0.503	0.392	0.368
3	0.735	0.568	0.369	0.520	0.501
4	0.679	0.582	0.507	0.409	0.637
5	1.008	0.908	0.908	0.626	0.912
6	1.163	0.914	0.926	0.750	1.251
7	1.457	0.973			1.453

	Standard Error									
	Lower					Upper				
Day	1:10	1:20	1:30	1:40	Control	1:10	1:20	1:30	1:40	Control
0	0.426	0.186	0.154	0.095	0.091	0.986	0.430	0.358	0.220	0.210
1	0.338	0.268	0.222	0.192	0.072	0.782	0.620	0.514	0.445	0.167
2	0.449	0.344	0.286	0.223	0.209	1.039	0.795	0.662	0.516	0.484
3	0.418	0.323	0.210	0.295	0.285	0.968	0.748	0.486	0.684	0.659
4	0.386	0.331	0.288	0.232	0.362	0.893	0.766	0.667	0.538	0.838
5	0.573	0.516	0.516	0.356	0.518	1.326	1.195	1.194	0.824	1.199
6	0.661	0.519	0.526	0.426	0.711	1.530	1.202	1.219	0.987	1.646
7	1.384	0.925			1.319	27.886	18.626			14.228

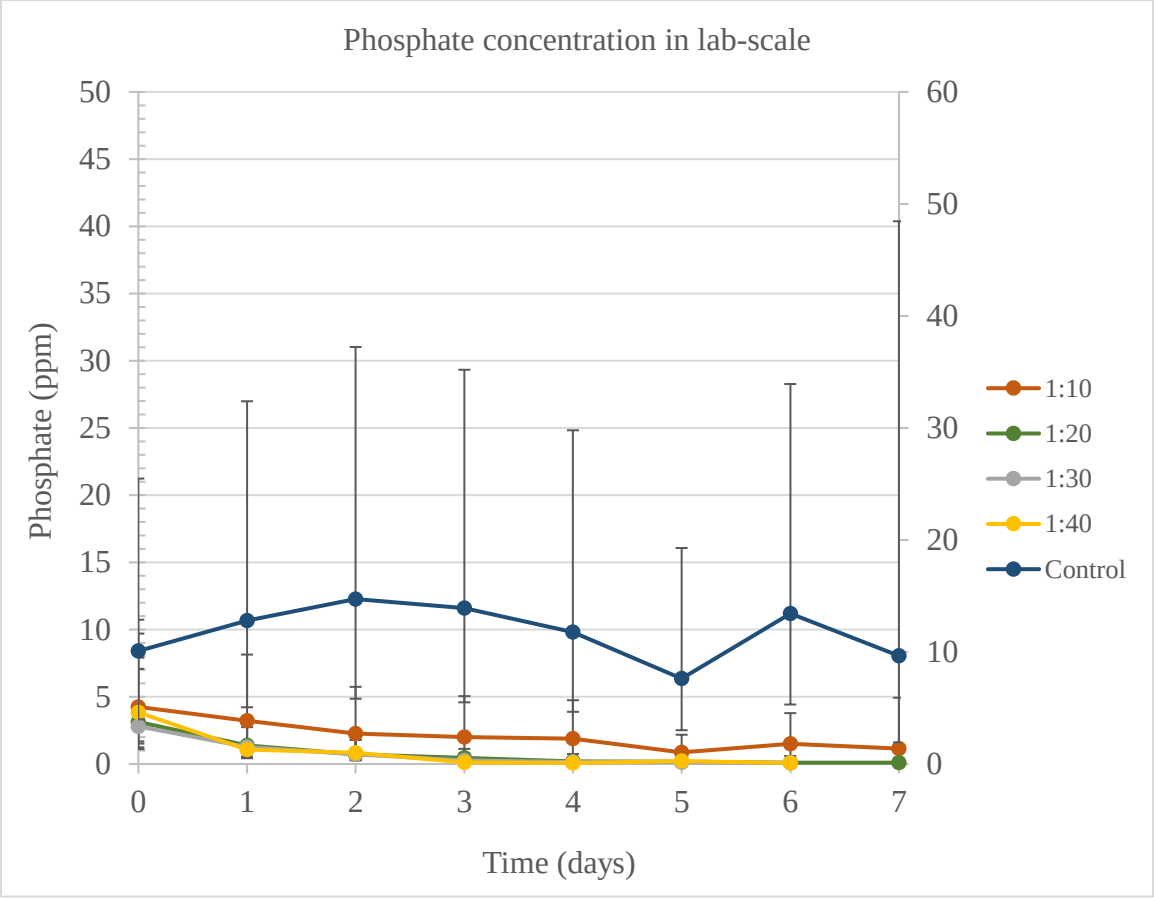

Figure 3. Daily phosphate concentration for the lab-scale during the different ratios of DW in water experiments. The primary vertical axis (on the left) is correspondent to the results for the ratios 1:10, 1:20, 1:30, and 1:40. The secondary vertical axis (on the right) is correspondent to the results for the control.

Table 5. Least square means for the phosphate concentration in lab-scale during the experiment to find the best ratio of DW in water (n = 20).

	Estimate				
	Ratio				
Day	1:10	1:20	1:30	1:40	Control
0	4.246	3.129	2.799	3.837	10.077
1	3.219	1.390	1.253	1.084	12.806
2	2.270	0.708	0.715	0.843	14.724
3	1.996	0.444	0.264	0.126	13.920
4	1.876	0.197	0.138	0.081	11.781
5	0.861	0.176	0.119	0.208	7.626
6	1.496	0.098	0.073	0.072	13.415
7	1.141	0.106			9.663

	Standard Error									
	Lower					Upper				
Day	1:10	1:20	1:30	1:40	Control	1:10	1:20	1:30	1:40	Control
0	2.567	1.891	1.692	2.319	6.092	6.489	4.783	4.279	5.865	15.403
1	1.946	0.841	0.758	0.655	7.741	4.921	2.125	1.916	1.657	19.576
2	1.372	0.428	0.432	0.510	8.901	3.470	1.082	1.093	1.288	22.505
3	1.207	0.269	0.160	0.076	8.415	3.051	0.679	0.404	0.193	21.275
4	1.134	0.119	0.084	0.058	7.122	2.868	0.301	0.212	0.206	18.010
5	0.520	0.106	0.099	0.126	4.610	1.315	0.269	0.590	0.318	11.655
6	0.905	0.059	0.044	0.047	8.110	2.287	0.149	0.111	0.136	20.504
7	0.877	0.081			7.736	3.789	0.351			38.788

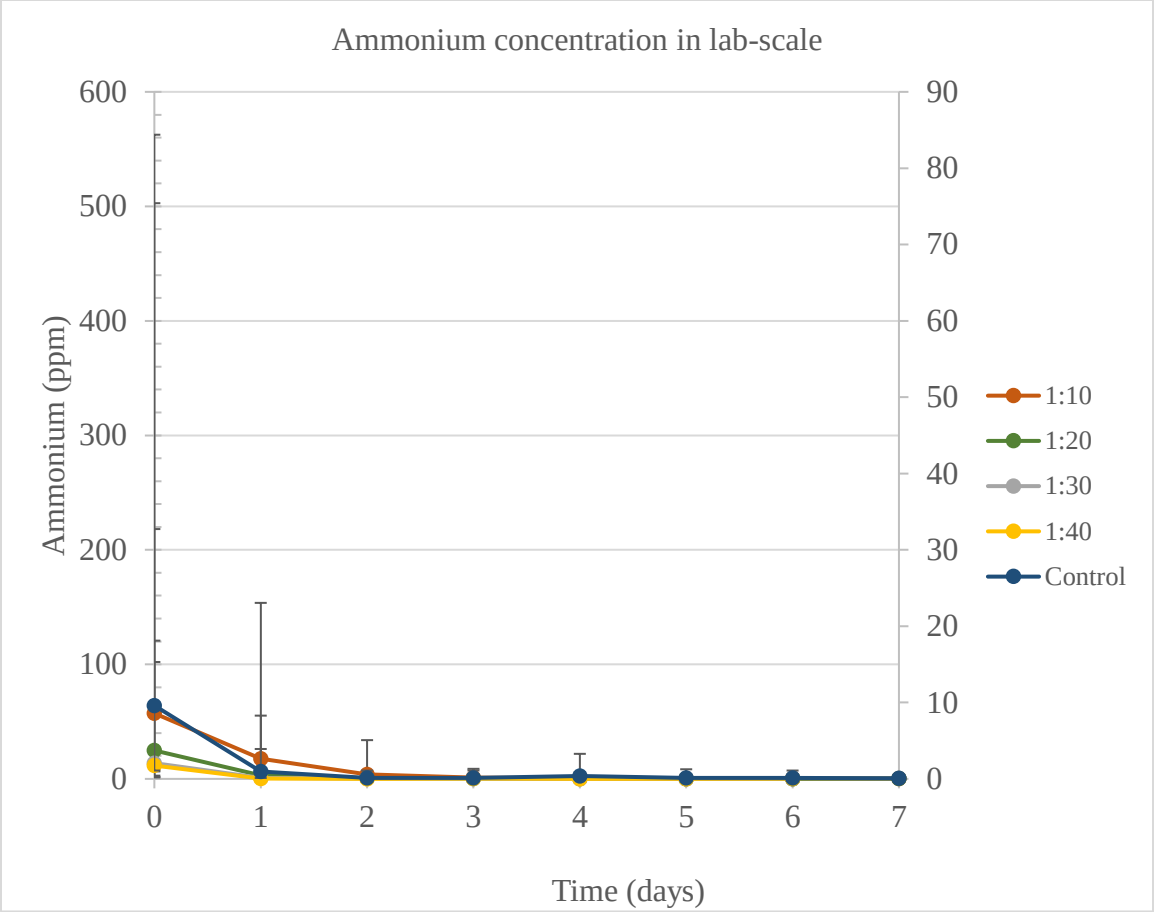

Figure 4. Ammonium concentration for the lab-scale experiment when evaluating the better ratio of DW in water. The primary vertical axis (on the left) is correspondent to the results for ratios 1:10, 1:20, 1:30, and 1:40. The secondary vertical axis (on the right) is correspondent to the results for the control.

Table 6. Least square means for the Ammonium concentration in lab-scale during the experiment to find the best ratio of DW in water (n = 20).

	Estimate				
	Ratio				
Day	1:10	1:20	1:30	1:40	Control
0	57.237	24.836	13.745	11.621	9.607
1	17.498	2.971	0.729	0.406	0.943
2	3.853	0.542	0.188	0.118	0.124
3	1.000	0.250	0.171	0.181	0.125
4	0.657	0.262	0.138	0.120	0.373
5	0.206	0.134	0.113	0.089	0.143
6	0.234	0.131	0.341	0.350	0.125
7	0.269	0.157			0.075

	Standard Error									
	Lower					Upper				
Day	1:10	1:20	1:30	1:40	Control	1:10	1:20	1:30	1:40	Control
0	50.721	22.009	12.180	10.298	8.513	445.517	193.339	106.990	90.453	74.779
1	15.506	2.633	0.646	0.360	0.836	136.201	23.128	5.678	3.164	7.342
2	3.415	0.480	0.167	0.104	0.110	29.992	4.219	1.467	0.918	0.964
3	0.886	0.222	0.152	0.160	0.111	7.781	1.947	1.332	1.405	0.976
4	0.582	0.232	0.122	0.107	0.331	5.116	2.042	1.071	0.937	2.907
5	0.182	0.119	0.100	0.079	0.127	1.601	1.041	0.878	0.693	1.114
6	0.207	0.116	0.302	0.310	0.110	1.822	1.020	2.653	2.727	0.970
7	0.050	0.029			0.015	0.061	0.036			0.018

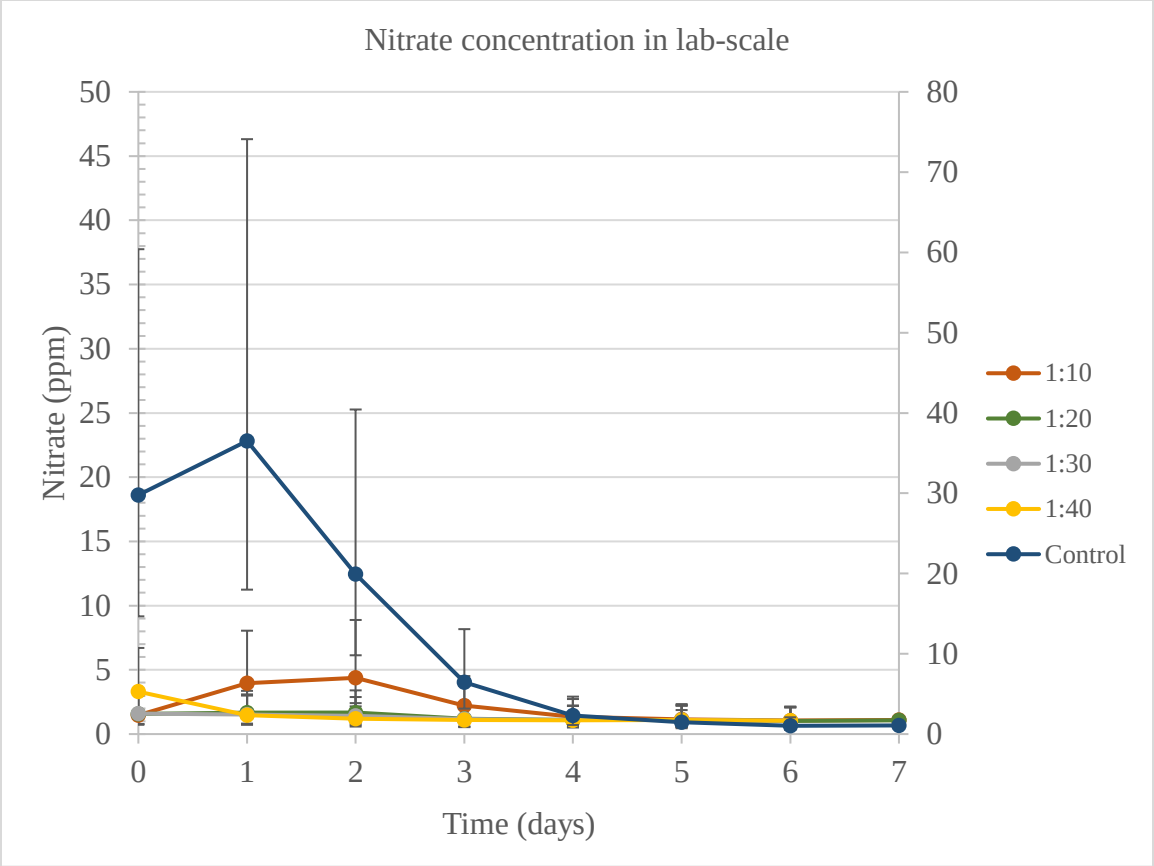

Figure 5. Nitrate concentration for the lab-scale experiment when evaluating the better ratio of DW in water. The primary vertical axis (on the left) is correspondent to the results for ratios 1:10, 1:20, 1:30, and 1:40. The secondary vertical axis (on the right) is correspondent to the results for the control.

Table 7. Least square means for the Nitrate concentration in lab-scale during the experiment to find the best ratio of DW in water (n = 20).

Estimate					
	Ratio				
Day	1:10	1:20	1:30	1:40	Control
0	1.469	1.580	1.596	3.304	29.761
1	3.967	1.658	1.522	1.477	36.514
2	4.378	1.677	1.420	1.187	19.922
3	2.225	1.202	1.163	1.095	6.441
4	1.344	1.086	1.092	1.076	2.298
5	1.150	1.107	1.085	1.104	1.474
6	1.058	1.021	1.030	1.035	1.032
7	1.096	1.067			1.085

Standard Error										
	Lower					Upper				
Day	1:10	1:20	1:30	1:40	Control	1:10	1:20	1:30	1:40	Control
0	0.745	0.801	0.810	1.676	15.096	1.512	1.626	1.643	3.401	30.640
1	2.012	0.841	0.772	0.749	18.524	4.082	1.707	1.567	1.521	37.592
2	2.221	0.851	0.721	0.602	10.106	4.507	1.726	1.462	1.222	20.510
3	1.129	0.610	0.590	0.556	3.267	2.291	1.238	1.197	1.128	6.631
4	0.682	0.551	0.554	0.546	1.166	1.384	1.118	1.125	1.108	2.366
5	0.583	0.562	0.550	0.560	0.748	1.184	1.140	1.117	1.137	1.518
6	0.537	0.518	0.522	0.525	0.523	1.089	1.051	1.060	1.066	1.062
7	0.160	0.164			0.103	0.187	0.194			0.114

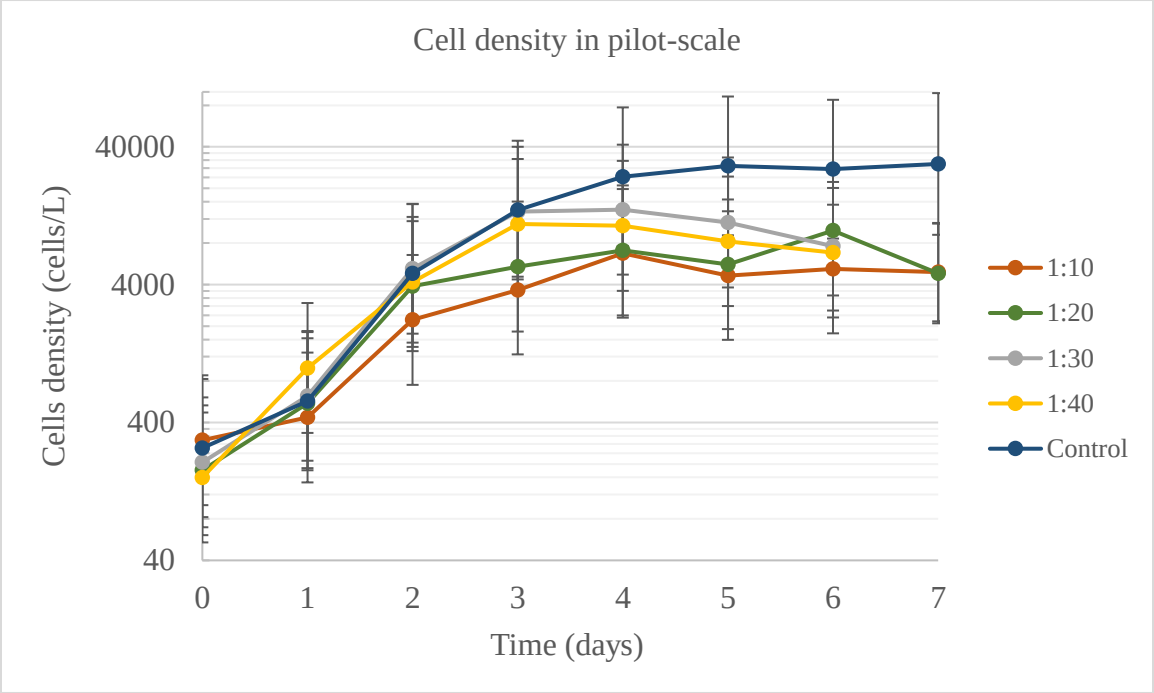

Figure 6. Cell density in pilot-scale during the experiment to find the best ratio of DW in water.

Table 8. Least square means for cell density in pilot-scale during the experiment to find the best ratio of DW in water (n = 36).

	Estimate				
	Ratio				
Day	1:10	1:20	1:30	1:40	Control
0	297.54	180.20	205.68	159.77	261.14
1	434.87	552.33	624.12	994.60	571.09
2	2218.85	3900.05	5214.74	4187.02	4826.34
3	3652.42	5408.05	13512.15	11015.35	13894.45
4	6744.24	7082.94	13992.05	10673.77	24280.89
5	4655.69	5619.76	11297.59	8244.86	29052.06
6	5190.81	9862.00	7594.24	6817.48	27505.60
7	4928.28	4820.07			30017.77

	Standard Error									
	Lower					Upper				
Day	1:10	1:20	1:30	1:40	Control	1:10	1:20	1:30	1:40	Control
0	196.95	119.24	136.15	105.76	178.74	582.66	352.44	402.72	312.87	566.48
1	287.78	365.62	412.81	658.42	390.95	850.81	1081.60	1219.42	1947.98	1239.57
2	1468.80	2582.74	3451.26	2772.64	3307.90	4345.08	7645.38	10205.63	8209.17	10514.05
3	2404.73	3581.21	8945.00	7293.99	9530.01	7040.58	10603.16	26464.25	21590.43	30343.78
4	4440.15	4691.04	9264.11	7068.89	16643.24	12994.62	13891.18	27416.58	20927.25	52902.90
5	3064.49	3716.08	7482.02	5448.58	19932.79	8966.36	10968.40	22153.67	16067.62	63501.68
6	3417.60	6524.95	4999.49	4505.30	18872.62	10004.54	19280.26	14630.13	13281.91	60130.19
7	2761.39	2723.74			20824.33	6279.29	6262.67			67984.39

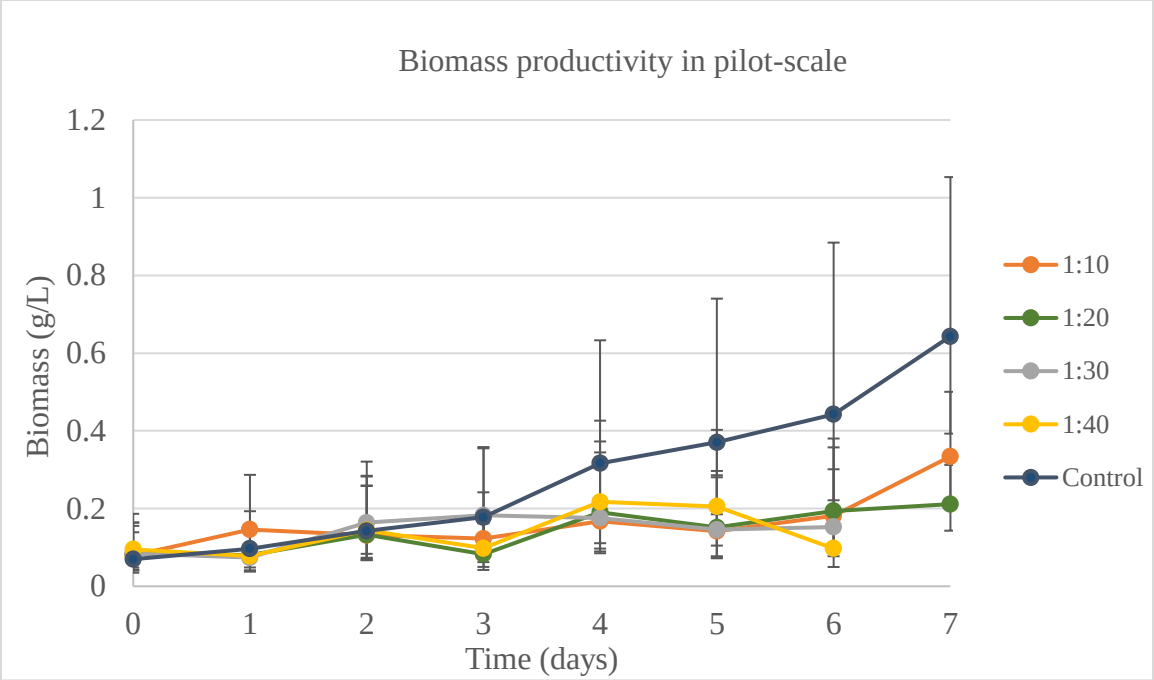

Figure 7. Biomass productivity per day of experiment on pilot-scale during the experiment to find the best ratio of DW in water.

Table 9. Least square means for biomass productivity during the experiment to find the best ratio of DW in water (n = 36).

	Estimate				
	Ratio				
Day	1:10	1:20	1:30	1:40	Control
0	0.0793	0.0835	0.0838	0.0951	0.0697
1	0.1463	0.0805	0.0741	0.0778	0.0968
2	0.1316	0.1323	0.1637	0.1441	0.1423
3	0.1226	0.0826	0.1828	0.0978	0.1776
4	0.1677	0.1902	0.1758	0.2174	0.3166
5	0.1422	0.1514	0.1458	0.2053	0.3704
6	0.1811	0.1940	0.1527	0.0974	0.4425
7	0.3341	0.2114			0.6432

	Standard Error									
	Lower					Upper				
Day	1:10	1:20	1:30	1:40	Control	1:10	1:20	1:30	1:40	Control
0	0.0389	0.0409	0.0411	0.0466	0.0348	0.0762	0.0802	0.0805	0.0914	0.0693
1	0.0717	0.0394	0.0363	0.0381	0.0483	0.1405	0.0773	0.0712	0.0748	0.0964
2	0.0645	0.0648	0.0802	0.0706	0.0711	0.1264	0.1271	0.1572	0.1384	0.1421
3	0.0605	0.0405	0.0895	0.0479	0.0887	0.1193	0.0794	0.1755	0.0940	0.1774
4	0.0827	0.0931	0.0861	0.1065	0.1582	0.1632	0.1825	0.1687	0.2088	0.3164
5	0.0701	0.0742	0.0714	0.1006	0.1851	0.1383	0.1454	0.1399	0.1972	0.3699
6	0.0894	0.0950	0.0753	0.0478	0.2211	0.1763	0.1863	0.1485	0.0936	0.4419
7	0.1111	0.0684			0.2504	0.1664	0.1010			0.4098

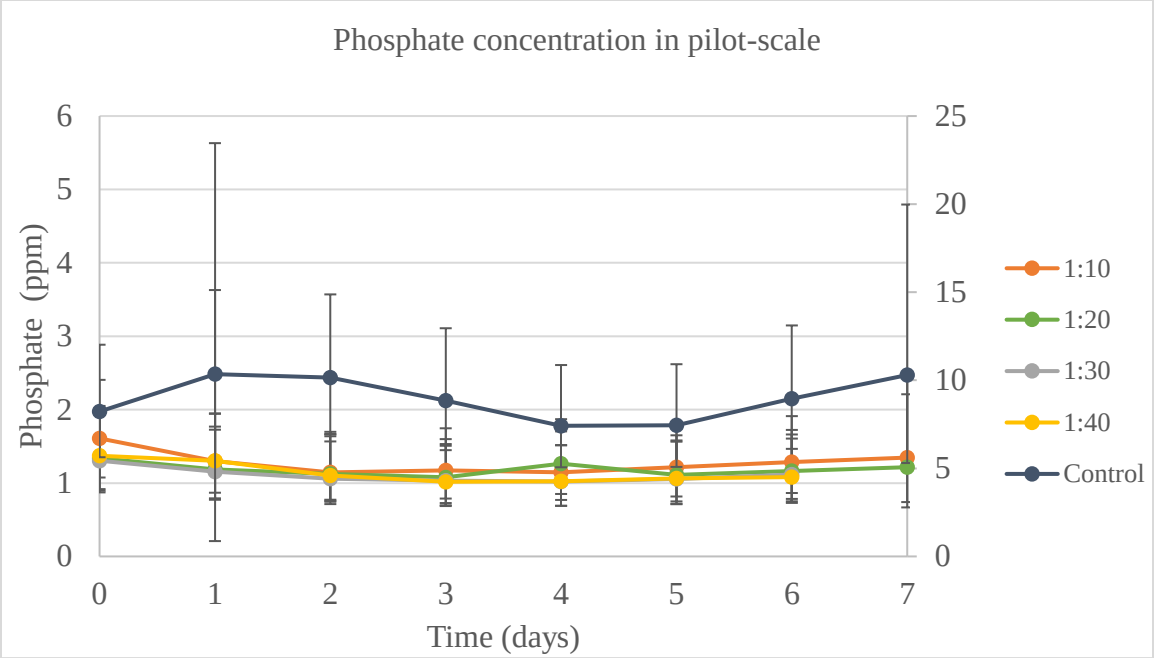

Figure 8. Daily phosphate concentration for the pilot-scale during the different ratios of DW in water experiments. The primary vertical axis (on the left) is correspondent to the results for ratios 1:10, 1:20, 1:30, and 1:40. The secondary vertical axis (on the right) is correspondent to the results for the control.

Table 10. Least square means for the phosphate concentration during the experiment to find the best ratio of DW in water (n = 36).

Estimate					
	Ratio				
Day	1:10	1:20	1:30	1:40	Control
0	1.608	1.338	1.304	1.371	8.227
1	1.298	1.183	1.155	1.303	10.348
2	1.145	1.125	1.057	1.104	10.151
3	1.173	1.078	1.033	1.017	8.841
4	1.145	1.262	1.021	1.023	7.416
5	1.215	1.112	1.055	1.065	7.448
6	1.285	1.163	1.117	1.082	8.950
7	1.346	1.215			10.292

Standard Error										
	Lower					Upper				
Day	1:10	1:20	1:30	1:40	Control	1:10	1:20	1:30	1:40	Control
0	0.533	0.443	0.432	0.454	2.596	0.797	0.662	0.646	0.679	3.792
1	0.430	0.392	0.383	0.432	3.267	0.643	0.586	0.572	0.645	4.775
2	0.373	0.366	0.344	0.359	3.224	0.552	0.543	0.510	0.533	4.724
3	0.385	0.351	0.336	0.331	2.808	0.572	0.520	0.498	0.491	4.113
4	0.375	0.411	0.332	0.333	2.355	0.558	0.609	0.493	0.494	3.449
5	0.398	0.363	0.343	0.347	2.365	0.593	0.538	0.509	0.515	3.465
6	0.421	0.379	0.366	0.353	2.842	0.627	0.561	0.545	0.524	4.165
7	0.606	0.547			4.990	1.102	0.995			9.685

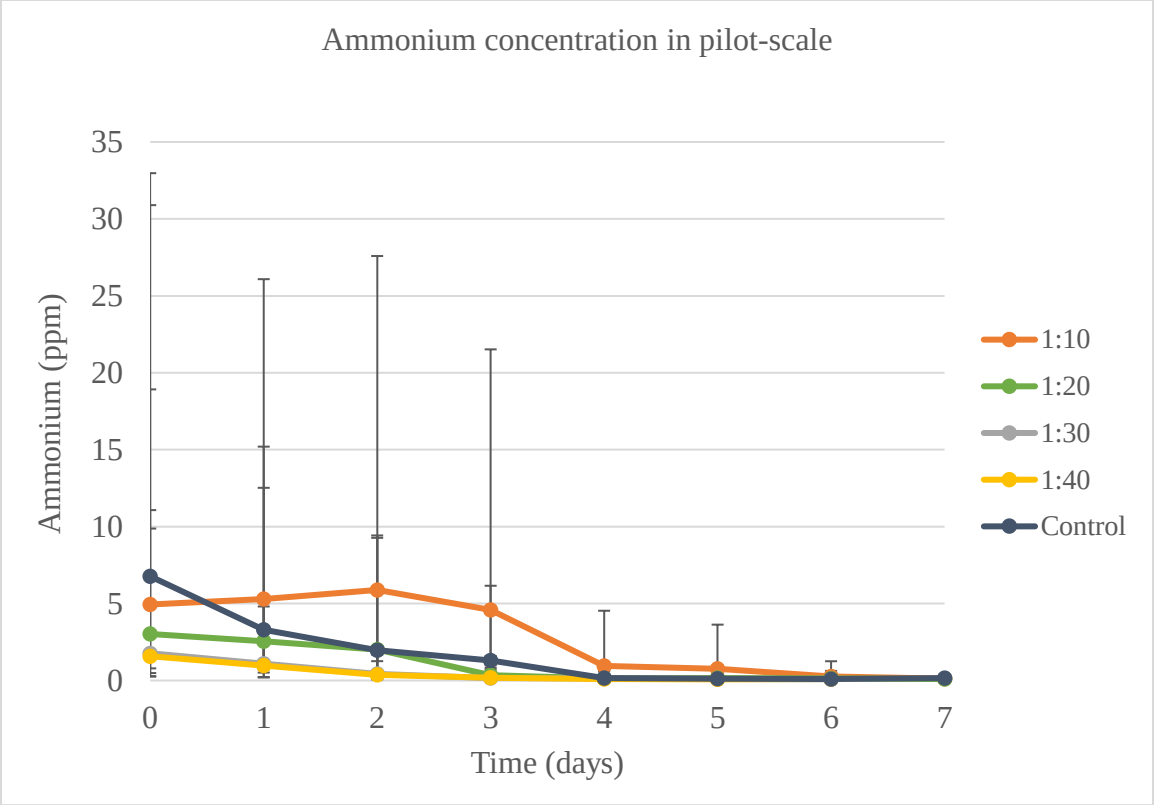

Figure 9. Ammonium concentration for the pilot-scale experiment when evaluating the better ratio of DW in water.

Table 11. Least square means for the Ammonium concentration during the experiment to find the best ratio of DW in water (n = 36).

Estimate					
	Ratio				
Day	1:10	1:20	1:30	1:40	Control
0	4.933	3.021	1.768	1.577	6.771
1	5.288	2.539	1.097	0.975	3.300
2	5.885	2.011	0.422	0.357	1.970
3	4.593	0.339	0.151	0.174	1.307
4	0.951	0.127	0.140	0.094	0.155
5	0.754	0.161	0.090	0.075	0.120
6	0.259	0.133	0.079	0.089	0.096
7	0.121	0.092			0.156

Standard Error										
	Lower					Upper				
Day	1:10	1:20	1:30	1:40	Control	1:10	1:20	1:30	1:40	Control
0	4.146	2.539	1.486	1.325	5.380	25.962	15.900	9.306	8.298	26.203
1	4.216	2.024	0.874	0.777	2.584	20.796	9.985	4.313	3.835	11.900
2	4.630	1.582	0.332	0.281	1.551	21.704	7.416	1.557	1.316	7.302
3	3.613	0.267	0.119	0.137	1.030	16.930	1.251	0.556	0.640	4.850
4	0.751	0.100	0.110	0.074	0.122	3.583	0.467	0.515	0.345	0.575
5	0.597	0.127	0.071	0.059	0.095	2.877	0.593	0.331	0.277	0.447
6	0.205	0.105	0.063	0.071	0.076	0.993	0.490	0.310	0.353	0.356
7	0.050	0.041			0.054	0.085	0.075			0.082

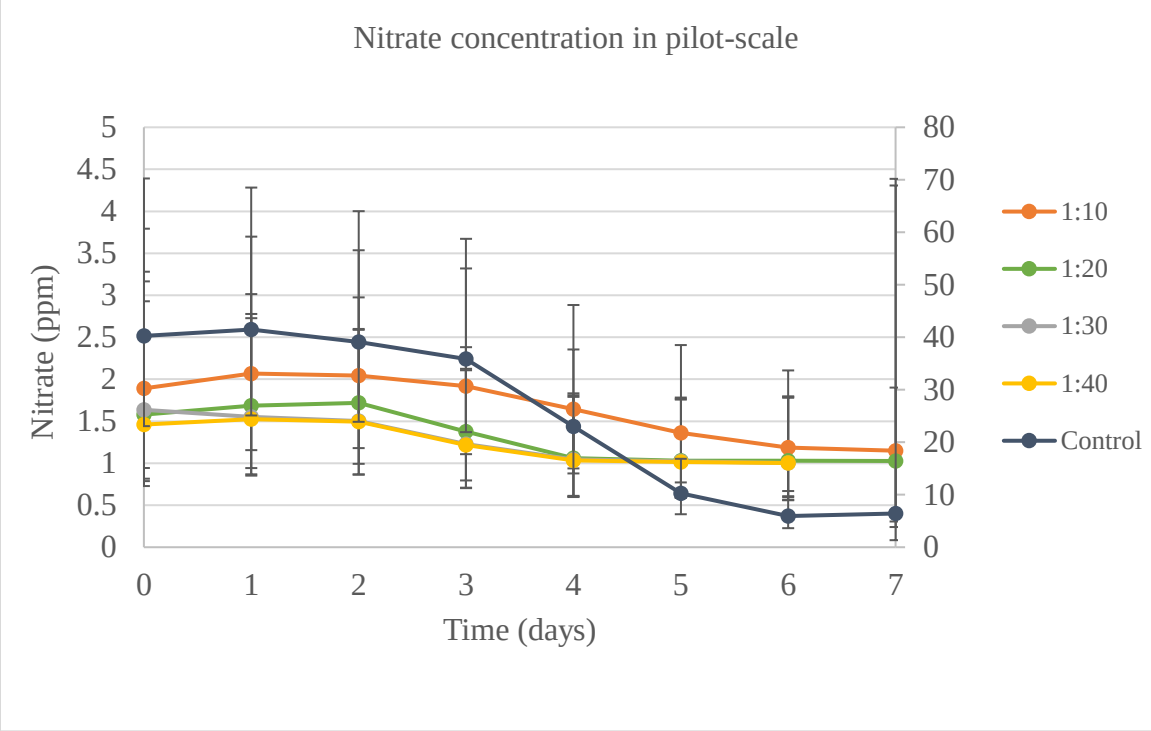

Figure 10. Nitrate concentration for the pilot-scale experiment when evaluating the better ratio of DW in water. The primary vertical axis (on the left) is correspondent to the results for ratios 1:10, 1:20, 1:30, and 1:40. The secondary vertical axis (on the right) is correspondent to the results for the control.

Table 12. Least square means for the Nitrate concentration during the experiment to find the best ratio of DW in water (n = 36).

Estimate					
	Ratio				
Day	1:10	1:20	1:30	1:40	Control
0	1.892	1.579	1.636	1.461	40.262
1	2.068	1.686	1.553	1.525	41.475
2	2.043	1.719	1.502	1.496	39.111
3	1.918	1.376	1.228	1.218	35.895
4	1.645	1.059	1.045	1.034	23.019
5	1.363	1.030	1.021	1.016	10.293
6	1.187	1.028	1.004	1.003	5.940
7	1.149	1.027			6.396

Standard Error										
	Lower					Upper				
Day	1:10	1:20	1:30	1:40	Control	1:10	1:20	1:30	1:40	Control
0	0.948	0.791	0.820	0.732	17.190	1.901	1.587	1.645	1.468	29.991
1	0.912	0.743	0.685	0.672	16.370	1.630	1.329	1.224	1.202	27.043
2	0.863	0.726	0.634	0.632	15.218	1.493	1.256	1.097	1.093	24.916
3	0.810	0.581	0.518	0.514	13.968	1.402	1.006	0.897	0.890	22.861
4	0.707	0.447	0.441	0.437	8.958	1.239	0.774	0.764	0.756	14.660
5	0.591	0.435	0.431	0.429	4.006	1.044	0.752	0.746	0.742	6.556
6	0.518	0.434	0.442	0.442	2.312	0.918	0.751	0.791	0.790	3.783
7	0.843	0.786			5.051	3.158	3.359			24.018

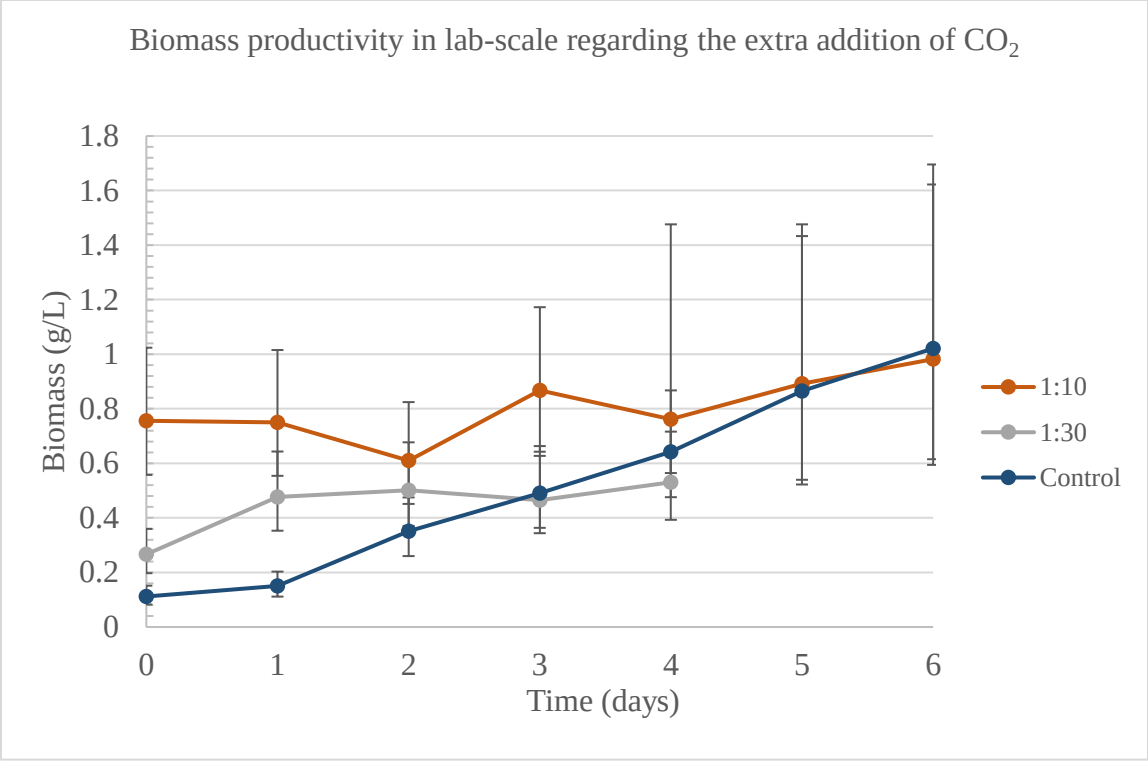

Figure 11. Biomass productivity for the lab-scale experiment when evaluating the significance in the extra addition of CO_2 using three different ratios.

Table 13. Least square means for biomass productivity in lab-scale when evaluating the significance in the extra addition of CO_2 using three different ratios (n = 24).

	Estimate			Standard Error					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	0.756	0.266	0.112	0.198	0.069	0.029	0.268	0.094	0.040
1	0.750	0.477	0.150	0.196	0.124	0.039	0.265	0.167	0.053
2	0.610	0.501	0.351	0.159	0.130	0.091	0.215	0.176	0.124
3	0.868	0.464	0.491	0.225	0.121	0.127	0.304	0.163	0.172
4	0.762	0.530	0.643	0.198	0.138	0.167	0.714	0.186	0.225
5	0.893		0.865	0.353		0.343	0.584		0.568
6	-6.926		1.021	-7.520		0.406	8.548		0.674

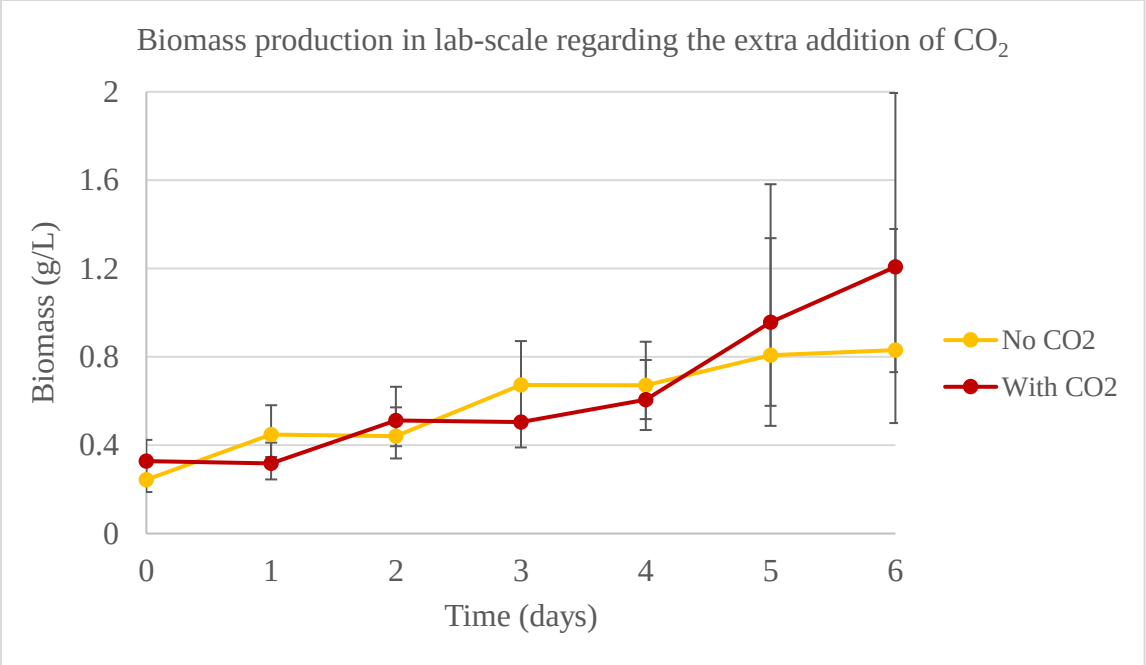

Figure 12. Biomass productivity for the lab-scale experiment when evaluating the significance of adding extra CO_2.

Table 14. Least square means for biomass productivity in lab-scale when evaluating the significance of adding extra CO_2 (n = 24).

	Estimate		Standard Error			
	Ratio		Lower		Upper	
Day	No	Yes	No	Yes	No	Yes
0	0.244	0.327	0.056	0.075	0.072	0.097
1	0.449	0.318	0.102	0.073	0.132	0.094
2	0.441	0.513	0.101	0.117	0.131	0.152
3	0.673	0.505	0.153	0.115	0.199	0.149
4	0.671	0.607	0.153	0.138	0.198	0.179
5	0.808	0.956	0.320	0.378	0.530	0.625
6	0.831	1.208	0.330	0.477	0.548	0.787

Table 15. Means for phosphate concentration in lab-scale for experiment 1 and 2, when evaluating the significance in the extra addition of CO_2 (n = 24).

Experiment 1						
	Extra CO_2			No extra CO_2		
Day	1:10	1:30	Control	1:10	1:30	Control
0	5.75	5.34	12.49	4.41	4.34	11.53
1	6.01	3.26	15.26	6.37	2.84	14.80
2	6.81	1.41	21.23	6.79	1.44	17.45
3	5.88	1.49	20.85	5.49	0.61	24.18
4	3.72	0.48	23.54	3.11	0.06	24.69
5	3.99		19.70	1.45		9.87
6	3.54		20.71	1.64		20.65
Experiment 2						
	Extra CO_2			No extra CO_2		
Day	1:10	1:30	Control	1:10	1:30	Control
0	12.57	5.92	33.73	7.68	7.28	32.79
1	6.98	2.01	43.06	1.81	2.84	37.74
2	3.90	1.54	43.15	2.65	1.27	43.24
3	2.82	0.26	38.47	1.63	0.94	15.47
4	4.00	0.23	29.91	1.40	0.94	41.62
5	2.41		25.64	1.99		39.35
6	2.95		35.37	4.34		38.83

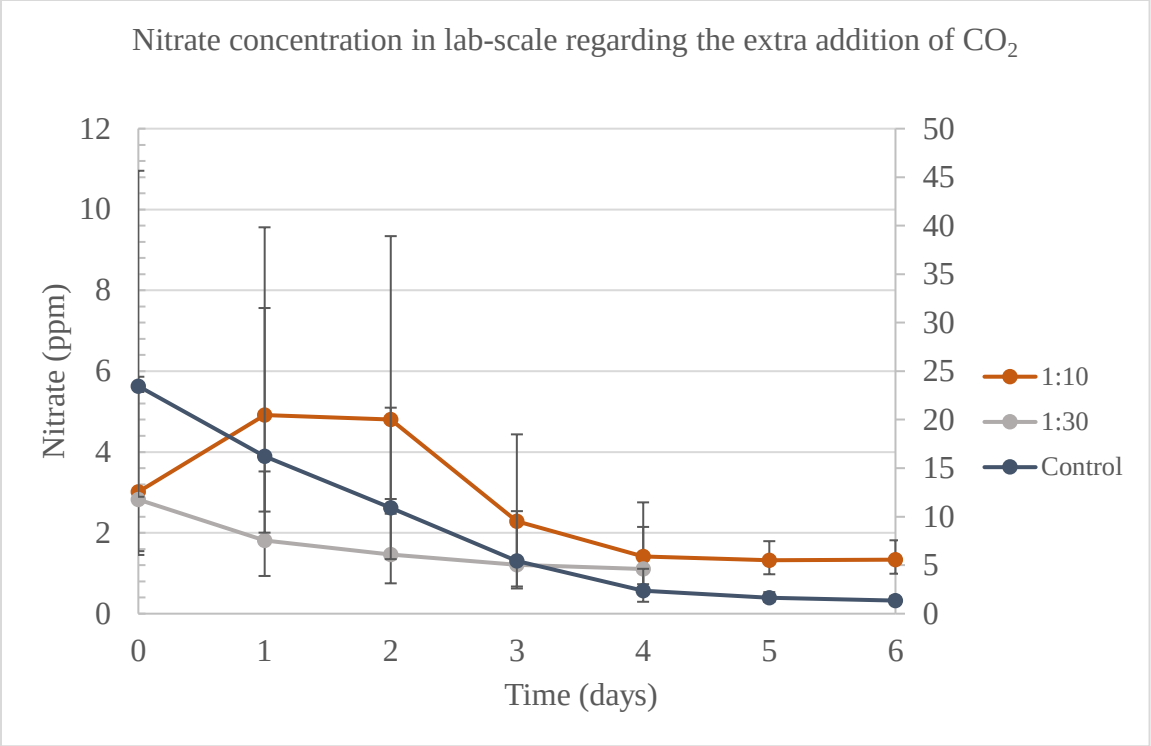

Figure 13. Results for the nitrate concentration for the lab-scale experiment when evaluating the significance in the extra addition of CO_2 using three different ratios. The results correspondent to the control is found on the secondary axis (on the right), and the results for the ratios 1:10 and 1:30, are on the primary axis (on the left).

Table 16. Least square means for nitrate concentration in lab-scale when evaluating the significance in the extra addition of CO_2 using three different ratios (n = 24).

	Estimate			Standard Error					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	3.012	2.823	23.458	1.464	1.370	11.407	2.850	2.662	22.201
1	4.914	1.812	16.223	2.388	0.879	7.871	4.645	1.707	15.287
2	4.806	1.460	10.926	2.333	0.709	5.305	4.535	1.377	10.311
3	2.283	1.212	5.440	1.108	0.588	2.640	2.153	1.142	5.129
4	1.418	1.105	2.382	0.688	0.536	1.156	1.336	1.041	2.245
5	1.322		1.639	0.347		0.432	0.471		0.586
6	1.340		1.349	0.351		0.357	0.476		0.485

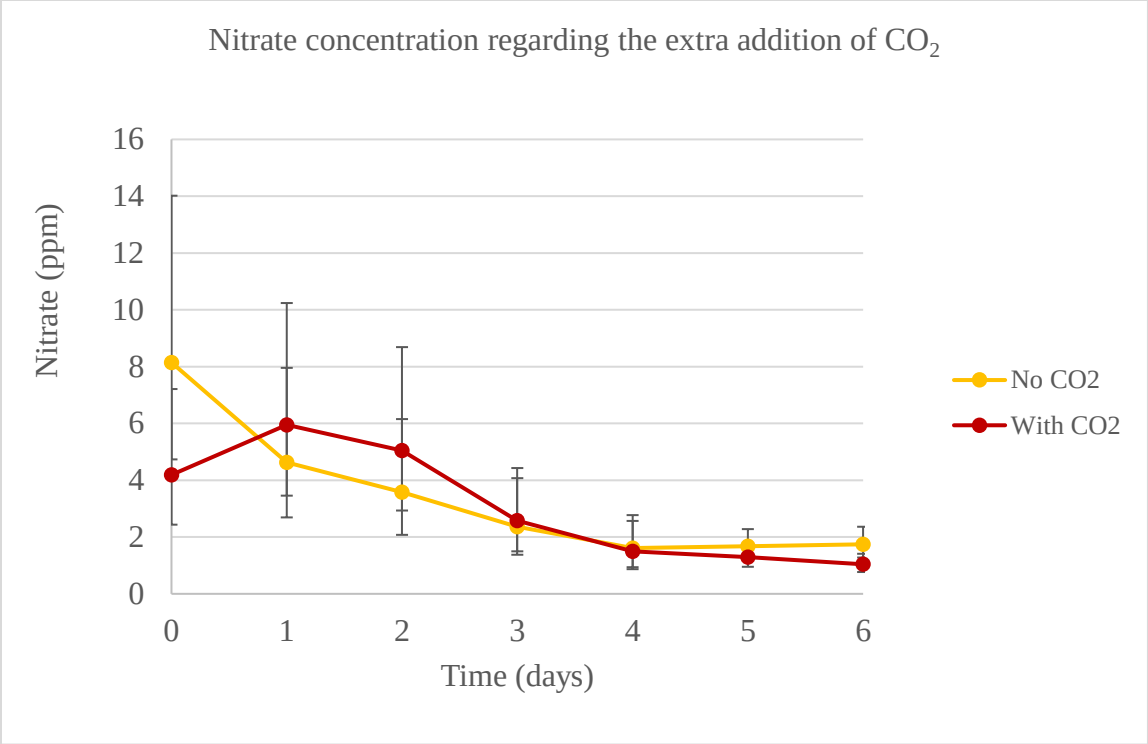

Figure 14. Nitrate concentration for the lab-scale experiment when evaluating the significance of adding extra CO_2.

Table 17. Least square means for nitrate concentration in lab-scale when evaluating the significance of adding extra CO_2 (n = 24).

	Estimate		Standard Error			
	Ratio		Lower		Upper	
Day	No	Yes	No	Yes	No	Yes
0	8.146	4.190	3.412	1.755	5.872	3.021
1	4.627	5.949	1.936	2.492	3.330	4.290
2	3.574	5.048	1.497	2.115	2.578	3.639
3	2.368	2.575	0.991	1.078	1.705	1.855
4	1.612	1.493	0.675	0.625	1.160	1.074
5	1.678	1.291	0.442	0.339	0.600	0.460
6	1.738	1.040	0.459	0.273	0.625	0.370

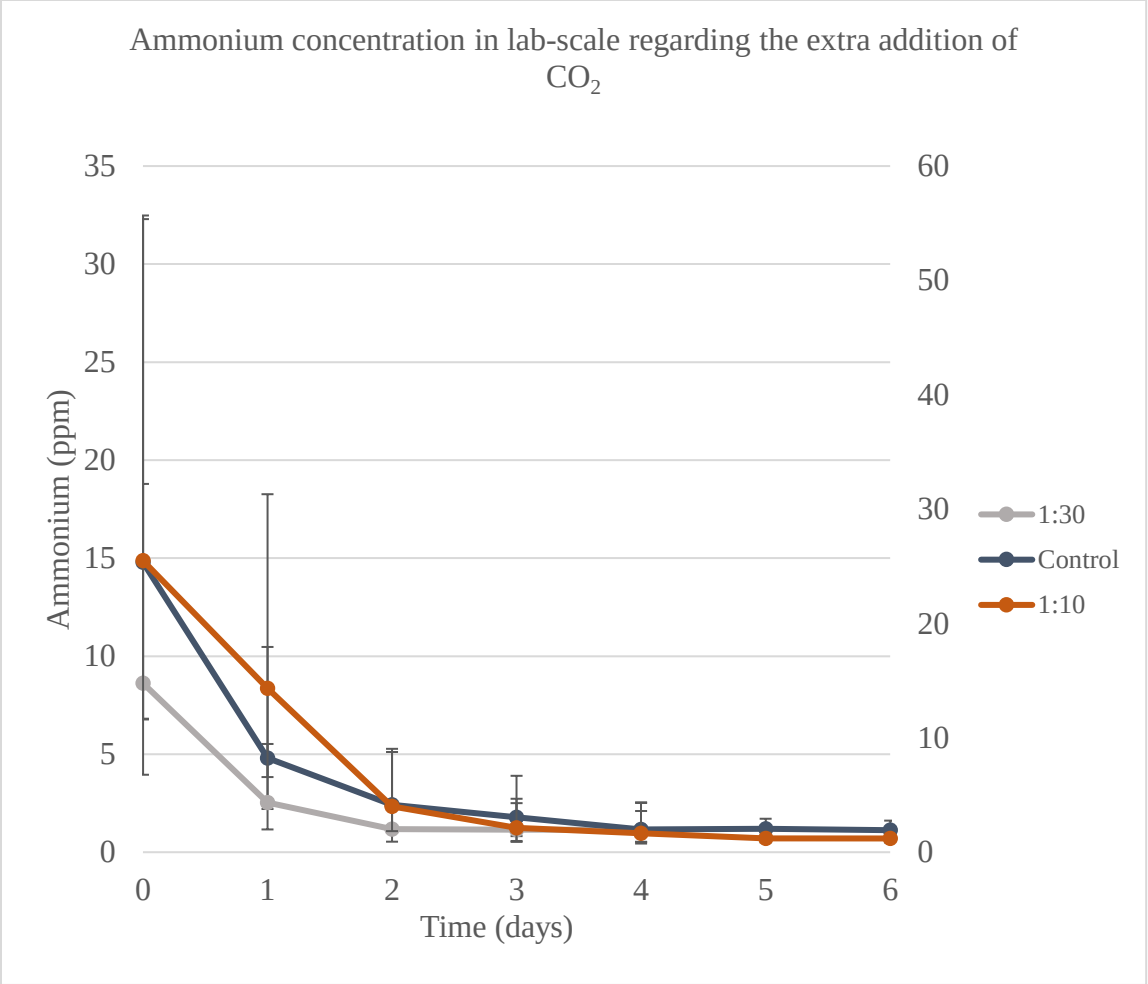

Figure 15. Ammonium concentration for the lab-scale experiment when evaluating the significance in the extra addition of CO_2 using three different ratios. The primary axis (on the left) is correspondent to the results for control and 1:30, while the secondary axis (on the right) corresponds to the results for 1:10.

Table 18. Least square means for ammonium concentration in lab-scale when evaluating the significance in the extra addition of CO_2 using three different ratios (n = 24).

	Estimate			Standard Error					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	25.498	8.618	14.788	13.821	4.665	8.016	30.181	10.170	17.507
1	14.345	2.534	4.805	7.772	1.371	2.600	16.961	2.988	5.666
2	4.018	1.174	2.419	2.176	0.635	1.310	4.745	1.386	2.856
3	2.144	1.149	1.788	1.161	0.622	0.968	2.531	1.355	2.110
4	1.653	1.152	1.157	0.895	0.624	0.631	1.950	1.359	1.389
5	1.213		1.192	0.366		0.360	0.525		0.516
6	1.203		1.124	0.363		0.340	0.520		0.488

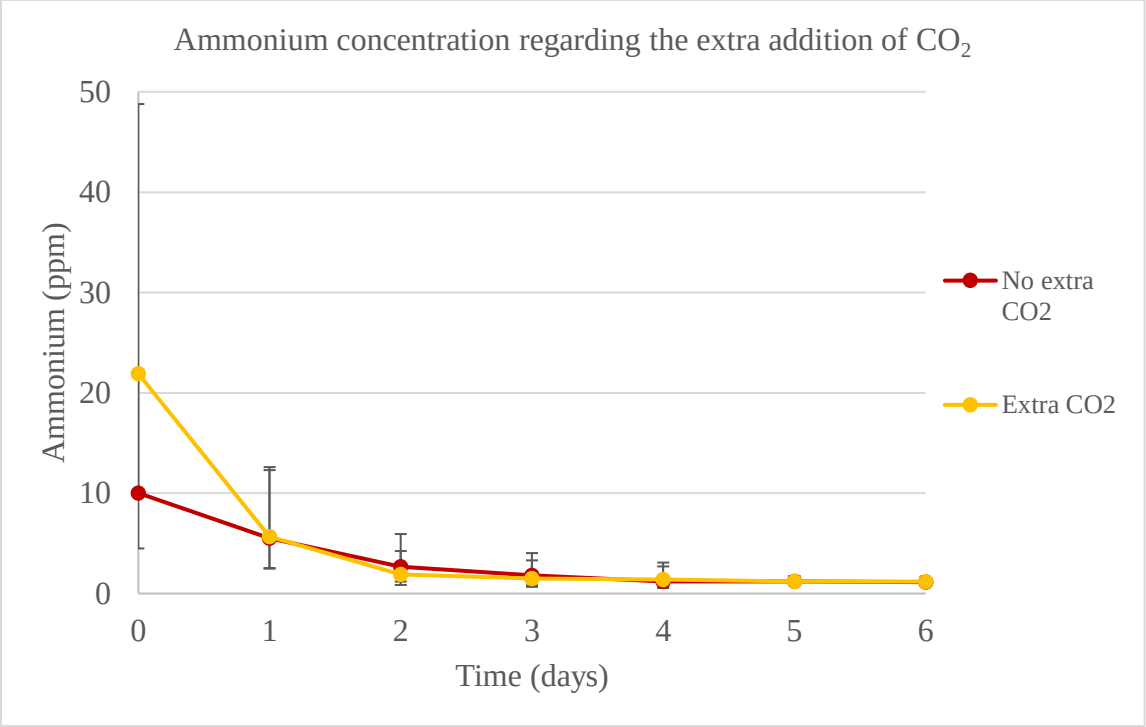

Figure 16. Ammonium concentration for the lab-scale experiment when evaluating the significance of adding extra CO_2.

Table 19. Least square means for ammonium concentration in lab-scale when evaluating the significance of adding extra CO_2 (n = 24).

	Estimate		Standard Error			
	Ratio		Lower		Upper	
Day	No	Yes	No	Yes	No	Yes
0	10.014	21.907	5.517	12.069	12.286	26.877
1	5.522	5.658	3.043	3.117	6.781	6.941
2	2.663	1.903	1.467	1.048	3.266	2.334
3	1.810	1.485	0.997	0.818	2.222	1.822
4	1.213	1.397	0.668	0.764	1.489	1.688
5	1.207	1.198	0.365	0.362	0.523	0.518
6	1.137	1.189	0.344	0.359	0.494	0.513

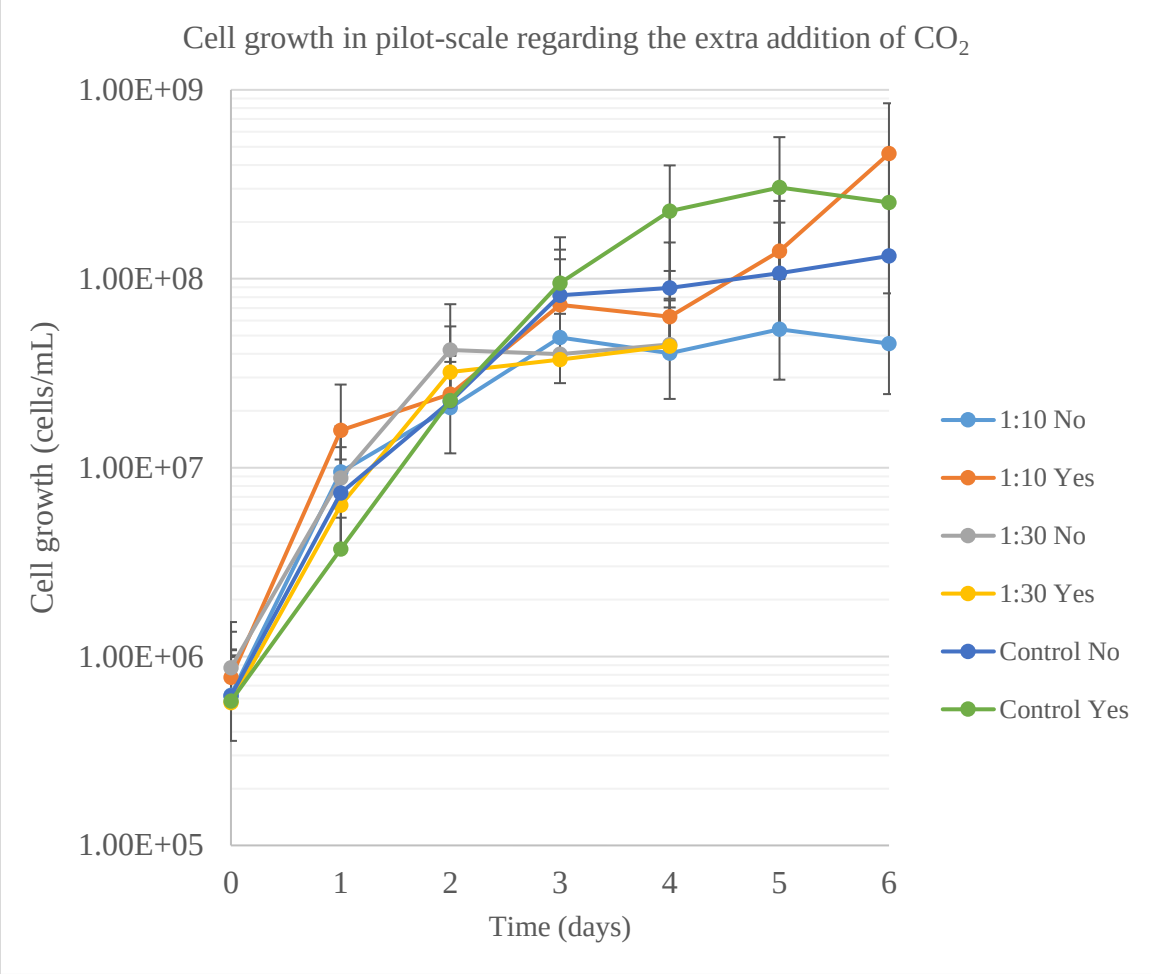

Figure 17. Cell density for the pilot-scale experiment when evaluating the significance on adding extra CO_2 using three different ratios.

Table 20. Least square means for cell density in pilot-scale when evaluating the significance on adding extra CO_2 using three different ratios (n = 12).

	Estimate for the ratios with extra CO_2			Standard Error for the ratios with extra CO_2					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	774598	570062	580938.4	-97188	-48406	223328	578084	425438	433555
1	1.6E+07	6324870	3707988	6960406	-1E+06	-2E+06	1.2E+07	4719154	2767276
2	2.4E+07	3.2E+07	22648405	-2E+07	9670175	1.1E+07	1.8E+07	2.4E+07	1.7E+07
3	7.3E+07	3.7E+07	94925222	3.3E+07	-4E+07	6.7E+07	5.4E+07	2.8E+07	7.1E+07
4	6.3E+07	4.4E+07	2.28E+08	1.8E+07	-5E+07	2E+08	4.7E+07	3.3E+07	1.7E+08
5	1.4E+08		3.04E+08	1.4E+08		2.7E+08	1.2E+08		2.6E+08
6	4.6E+08		2.53E+08	4.6E+08		2.3E+08	3.9E+08		2.1E+08
	Estimate for the ratios without extra CO_2			Standard Error for the ratios with extra CO_2					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	624496	871787	618467.5	-150102.542	301725	37529.2	466062.073	650615	461563
1	9486687	8802972	7348450	-6276691.69	2478103	3640462	7079926.66	6569670	5484158
2	2.1E+07	4.2E+07	22360353	-3711376.03	9957706	-288052	15511218.7	3.1E+07	1.7E+07
3	4.9E+07	4E+07	81670221	-23684150.2	2551381	-1E+07	36493331.3	3E+07	6.1E+07
4	4E+07	4.5E+07	89263212	-22615735.2	897837	-1E+08	30106152.1	3.4E+07	6.7E+07
5	5.4E+07		1.07E+08	-85882552.6		-2E+08	45748190.9		9.1E+07
6	4.5E+07		1.32E+08	-414292601		-1E+08	38369092.3		1.1E+08

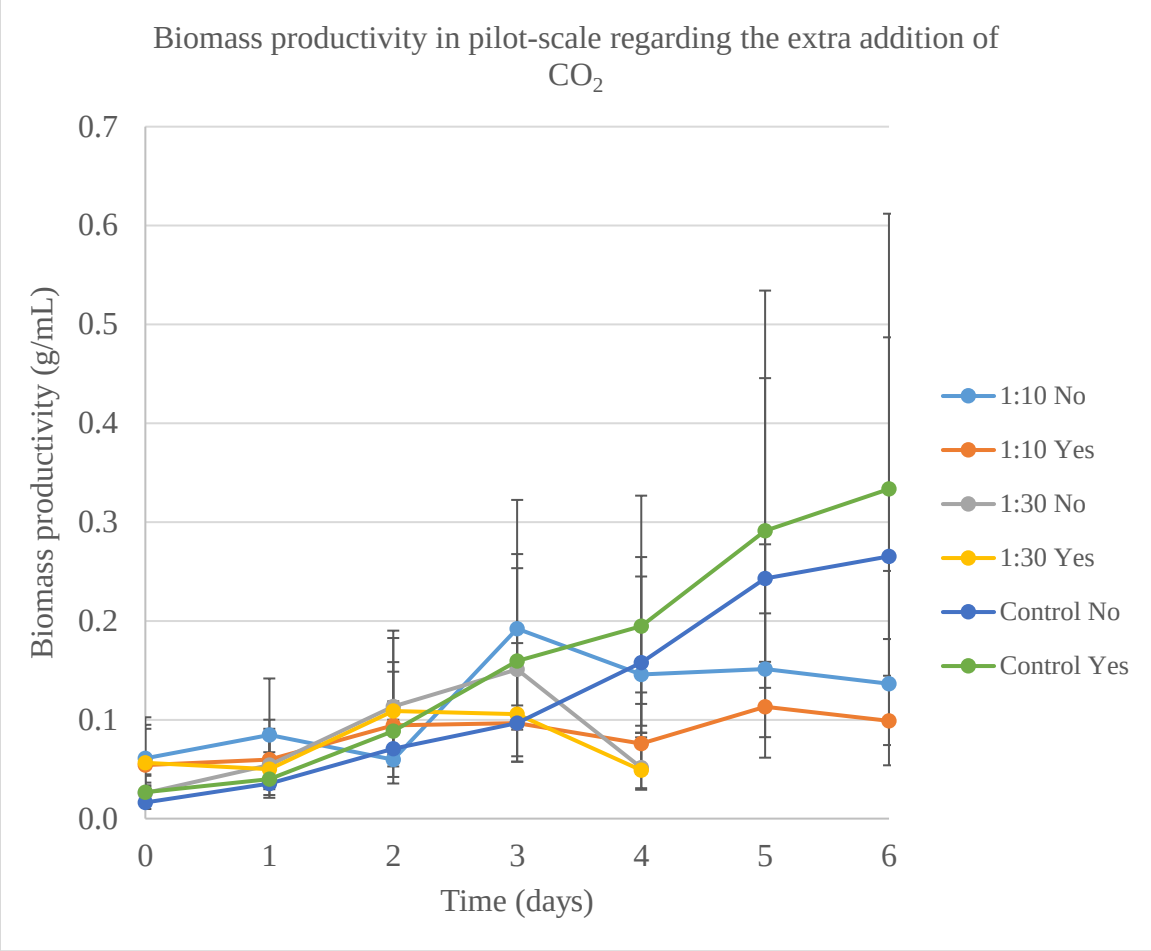

Figure 18. Biomass productivity for the pilot-scale experiment when evaluating the significance in the extra addition of CO_2 using three different ratios.

Table 21. Least square means for biomass productivity in pilot-scale when evaluating the significance in the extra addition of CO_2 using three different ratios (n = 12).

	Estimate for the ratios with extra CO_2			Standard Error for the ratios with extra CO_2					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	0.054	0.057	0.027	0.022	0.023	0.011	0.037	0.038	0.018
1	0.060	0.050	0.040	0.024	0.020	0.016	0.040	0.034	0.027
2	0.094	0.109	0.089	0.038	0.044	0.036	0.064	0.074	0.060
3	0.097	0.106	0.159	0.039	0.043	0.064	0.066	0.072	0.108
4	0.076	0.049	0.195	0.031	0.020	0.079	0.052	0.033	0.132
5	0.113		0.291	0.051		0.132	0.095		0.243
6	0.099		0.333	0.045		0.152	0.083		0.279
	Estimate for the ratios without extra CO_2			Standard Error for the ratios with extra CO_2					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	0.061	0.026	0.016	0.025	0.010	0.007	0.041	0.018	0.011
1	0.084	0.054	0.035	0.034	0.022	0.014	0.057	0.037	0.024
2	0.060	0.113	0.071	0.024	0.046	0.029	0.040	0.077	0.048
3	0.192	0.151	0.097	0.078	0.061	0.039	0.130	0.102	0.066
4	0.146	0.052	0.158	0.059	0.021	0.064	0.099	0.035	0.107
5	0.151		0.243	0.069		0.111	0.126		0.203
6	0.137		0.265	0.062		0.121	0.114		0.222

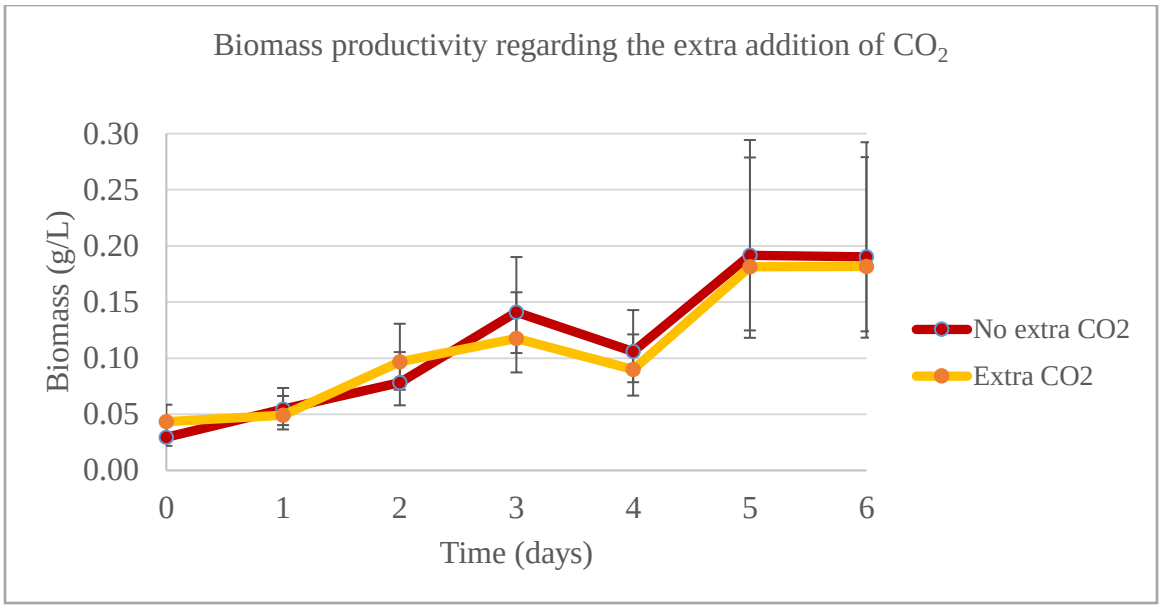

Figure 19. Biomass productivity for the pilot-scale experiment when evaluating the significance of adding extra CO_2.

Table 22. Least square means for biomass productivity in pilot-scale when evaluating the significance of adding extra CO_2 (n = 12).

	Estimate		Standard Error			
	Ratio		Lower		Upper	
Day	No	Yes	No	Yes	No	Yes
0	0.030	0.043	0.008	0.011	0.010	0.015
1	0.054	0.049	0.014	0.013	0.019	0.017
2	0.078	0.097	0.020	0.025	0.027	0.034
3	0.141	0.118	0.036	0.030	0.049	0.041
4	0.106	0.090	0.027	0.023	0.037	0.031
5	0.192	0.181	0.067	0.063	0.103	0.097
6	0.190	0.182	0.066	0.063	0.102	0.097

Table 23. Biomass weight when the entire bag with microalgae was harvested in the last day of experiment for each of the ratios and controls.

Biomass weight when extra CO_2 was added			
Treatment	Wet weight (g)	Dry weight (g)	Dry mass (%)
Control	341.13	42.31	12.40
1:10	119.58	10.60	8.86
1:30	39.40	5.07	12.87
Biomass weight when no extra CO_2 was added			
Treatment	Wet weight (g)	Dry weight (g)	Dry mass (%)
Control	109.68	12.85	11.71
1:10	136.37	10.07	7.38
1:30	20.36	2.93	14.39

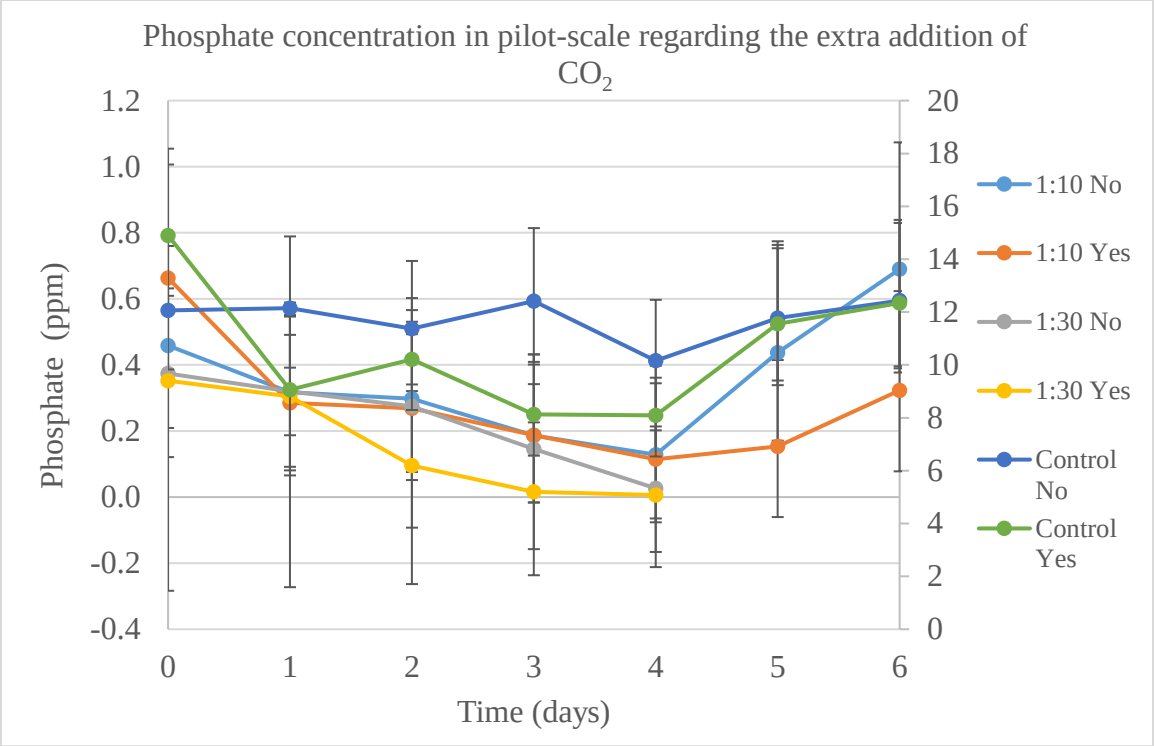

Figure 20. Phosphate concentration for the pilot-scale experiment when evaluating the significance in the extra addition of CO_2 using three different ratios. The primary axis (on the left) correspond to the results of the ratios, and the secondary axis (on the right) correspond to the results for the controls.

Table 24. Least square means phosphate concentration in pilot-scale when evaluating the significance in the extra addition of CO_2 using three different ratios (n = 12).

	Estimate for the ratios with extra CO_2			Standard Error for the ratios with extra CO_2					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	0.663	0.352	14.900	0.285	0.231	2.720	0.343	0.279	3.283
1	0.286	0.304	9.062	0.220	0.223	1.722	0.265	0.269	2.076
2	0.269	0.095	10.214	0.217	0.187	1.919	0.262	0.226	2.315
3	0.187	0.016	8.130	0.203	0.174	1.563	0.245	0.210	1.885
4	0.114	0.006	8.093	0.191	0.172	1.556	0.230	0.208	1.876
5	0.153		11.560	0.213		2.325	0.262		2.855
6	0.323		12.342	0.245		2.471	0.301		3.031
	Estimate for the ratios without extra CO_2			Standard Error for the ratios with extra CO_2					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	0.459	0.374	12.065	0.250	0.235	2.236	0.301	0.284	2.697
1	0.317	0.320	12.147	0.225	0.226	2.250	0.272	0.273	2.714
2	0.298	0.275	11.378	0.222	0.218	2.117	0.268	0.263	2.556
3	0.186	0.146	12.410	0.203	0.196	2.295	0.245	0.237	2.767
4	0.128	0.026	10.160	0.193	0.176	1.910	0.233	0.212	2.304
5	0.437		11.775	0.266		2.365	0.327		2.904
6	0.690		12.434	0.313		2.487	0.384		3.053

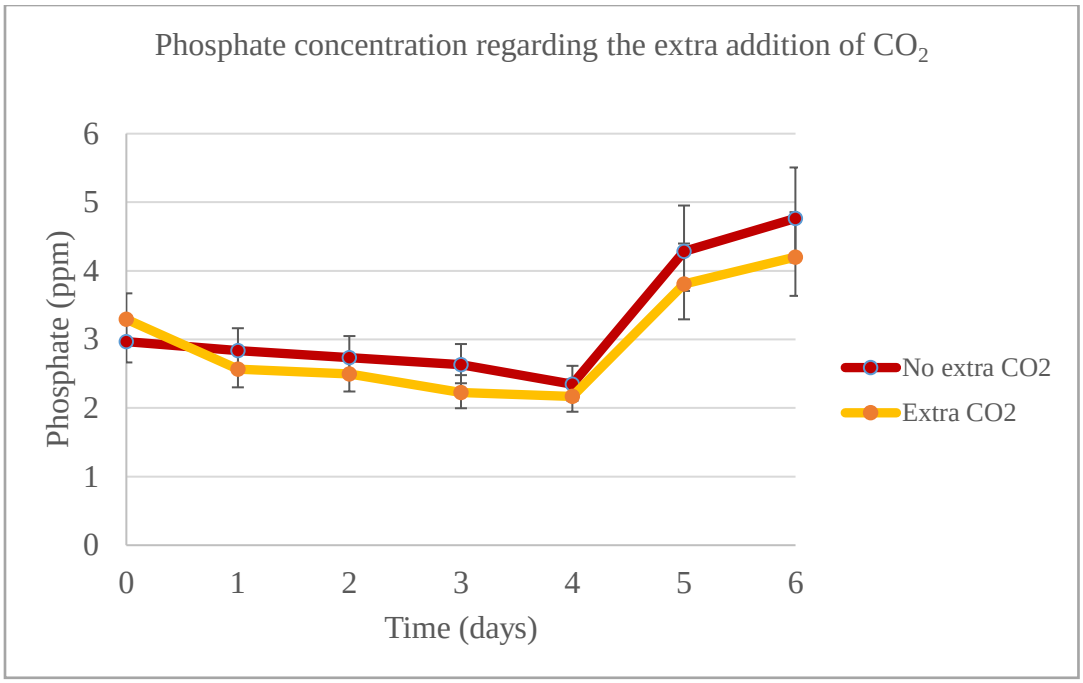

Figure 21. Phosphate concentration for the pilot-scale experiment when evaluating the significance on adding extra of CO_2.

Table 25. Least square means phosphate concentration in pilot-scale when evaluating the significance on adding extra of CO_2 (n = 12).

	Estimate		Standard Error			
	Ratio		Lower		Upper	
Day	No	Yes	No	Yes	No	Yes
0	2.970	3.295	0.305	0.338	0.340	0.377
1	2.838	2.565	0.291	0.263	0.325	0.294
2	2.736	2.498	0.281	0.257	0.313	0.286
3	2.632	2.225	0.270	0.229	0.301	0.255
4	2.347	2.168	0.241	0.223	0.268	0.248
5	4.284	3.806	0.578	0.513	0.668	0.593
6	4.765	4.201	0.642	0.566	0.742	0.654

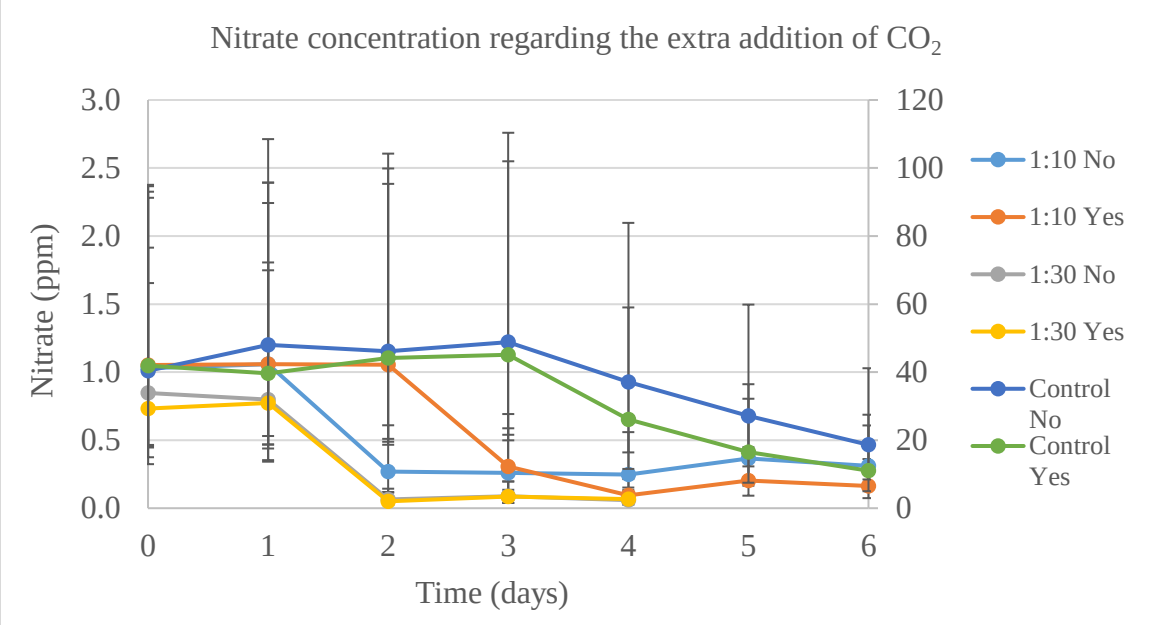

Figure 22. Nitrate concentration for the pilot-scale experiment when evaluating the significance of the extra addition of CO_2 using three different ratios. The primary axis (on the left) correspond to the results of the ratios, and the secondary axis (on the right) correspond to the results for the controls.

Table 26. Least square means for nitrate concentration in pilot-scale when evaluating the significance in the extra addition of CO_2 using three different ratios (n = 12).

	Estimate for the ratios with extra CO_2			Standard Error for the ratios with extra CO_2					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	1.051	0.732	41.863	0.586	0.408	23.348	1.325	0.923	52.788
1	1.058	0.773	39.678	0.590	0.431	22.129	1.335	0.975	50.033
2	1.054	0.051	44.168	0.588	0.028	24.633	1.329	0.064	55.695
3	0.306	0.087	45.105	0.171	0.048	25.156	0.386	0.109	56.877
4	0.095	0.067	26.104	0.053	0.037	14.559	0.119	0.085	32.917
5	0.203		16.517	0.111		9.034	0.245		19.935
6	0.164		11.025	0.090		6.030	0.198		13.309
	Estimate for the ratios without extra CO_2			Standard Error for the ratios with extra CO_2					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	1.029	0.847	40.358	0.574	0.472	22.508	1.297	1.068	50.891
1	1.058	0.799	47.986	0.590	0.445	26.762	1.334	1.007	60.509
2	0.270	0.063	46.099	0.150	0.035	25.708	0.340	0.080	58.131
3	0.260	0.087	48.813	0.145	0.049	27.224	0.328	0.110	61.553
4	0.247	0.059	37.096	0.138	0.033	20.687	0.312	0.074	46.777
5	0.364		27.123	0.199		14.835	0.440		32.736
6	0.311		18.644	0.170		10.197	0.376		22.505

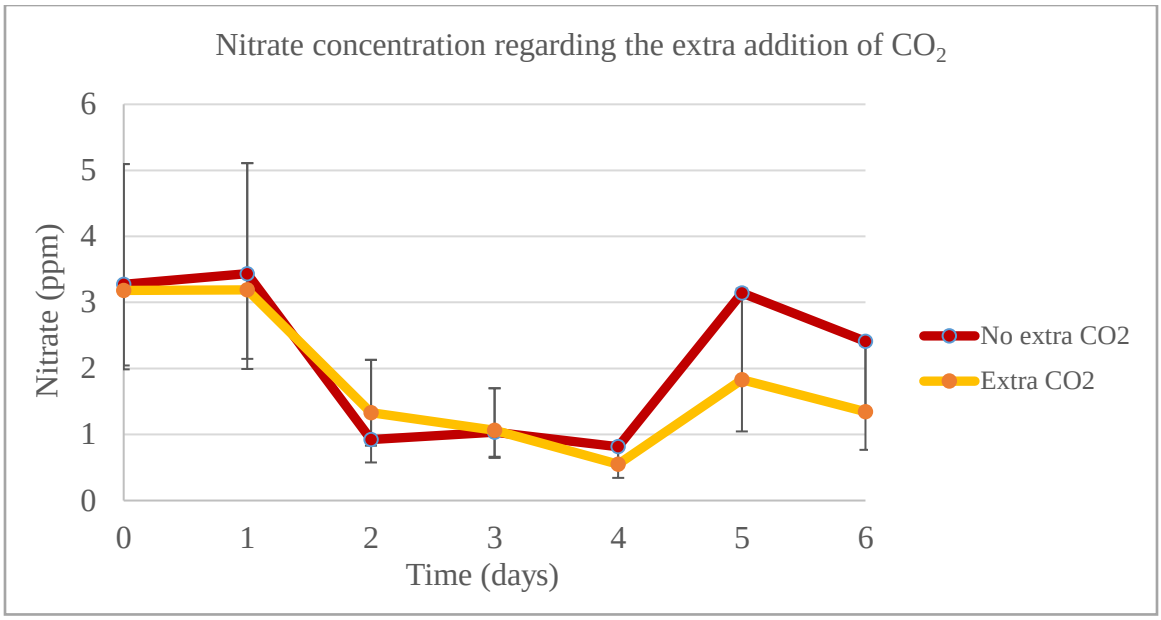

Figure 23. Nitrate concentration for the pilot-scale experiment when evaluating the significance of adding extra CO_2.

Table 27. Least square means for nitrate concentration in pilot-scale when evaluating the significance of adding extra CO_2 (n = 12).

	Estimate		Standard Error			
	Ratio		Lower		Upper	
Day	No	Yes	No	Yes	No	Yes
0	3.276	3.181	1.231	1.195	1.819	1.914
1	3.436	3.190	1.291	1.198	1.674	1.919
2	0.924	1.330	0.347	0.500	1.207	0.800
3	1.034	1.062	0.388	0.399	0.667	0.639
4	0.815	0.549	0.306	0.206	0.065	0.331
5	3.144	1.832	1.348	0.785	0.063	1.375
6	2.409	1.343	1.033	0.576	-0.058	1.008

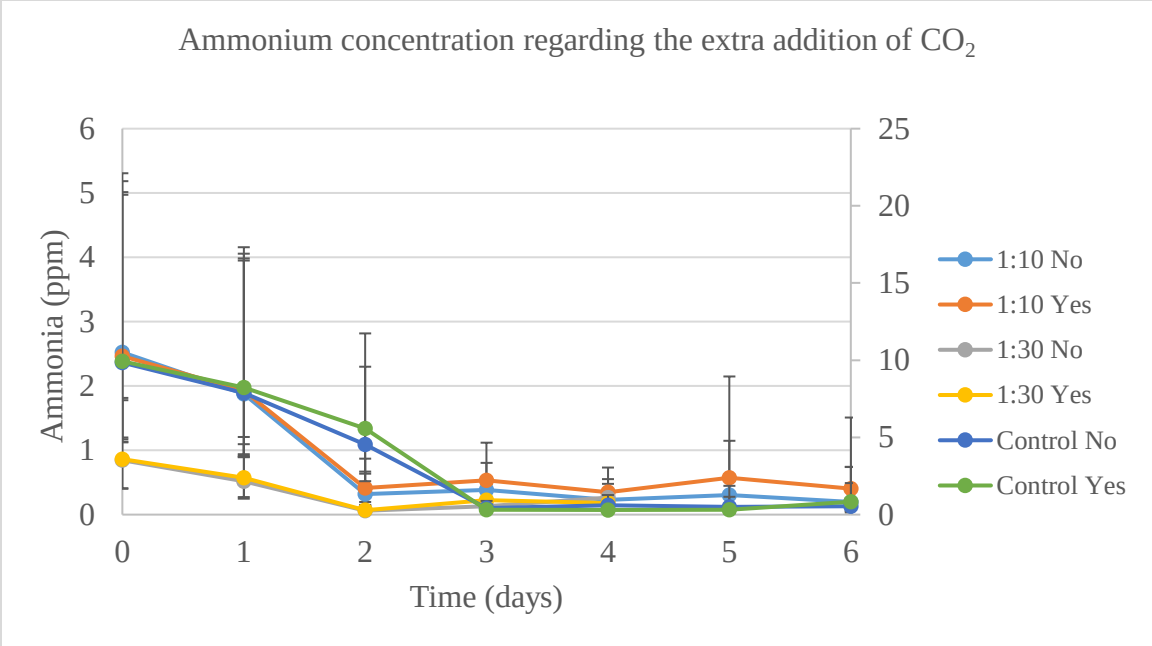

Figure 24. Ammonium concentration for the pilot-scale experiment when evaluating the significance in the extra addition of CO_2 using three different ratios. The primary axis (on the left) correspond to the results of the ratios, and the secondary axis (on the right) correspond to the results for the controls.

Table 28. Least square means for ammonium concentration in pilot-scale when evaluating the significance in the extra addition of CO_2 using three different ratios (n = 12).

	Estimate for the ratios with extra CO_2			Standard Error for the ratios with extra CO_2					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	2.464	0.862	9.929	1.293	0.452	5.210	2.720	0.952	10.961
1	1.928	0.574	8.232	1.012	0.301	4.320	2.129	0.633	9.087
2	0.413	0.068	5.578	0.217	0.036	2.927	0.456	0.075	6.159
3	0.531	0.225	0.316	0.279	0.118	0.166	0.587	0.249	0.349
4	0.348	0.180	0.301	0.182	0.094	0.158	0.384	0.198	0.332
5	0.572		0.307	0.420		0.225	1.575		0.846
6	0.402		0.825	0.295		0.605	1.107		2.270
	Estimate for the ratios without extra CO_2			Standard Error for the ratios with extra CO_2					
	Ratio			Lower			Upper		
Day	1:10	1:30	Control	1:10	1:30	Control	1:10	1:30	Control
0	2.522	0.845	9.846	1.324	0.444	5.166	2.784	0.933	10.870
1	1.877	0.520	7.888	0.985	0.273	4.139	2.073	0.574	8.709
2	0.318	0.061	4.553	0.167	0.032	2.389	0.351	0.067	5.027
3	0.382	0.132	0.422	0.201	0.069	0.221	0.422	0.146	0.466
4	0.227	0.262	0.602	0.119	0.138	0.316	0.251	0.290	0.665
5	0.306		0.497	0.224		0.364	0.842		1.367
6	0.197		0.544	0.145		0.399	0.542		1.497

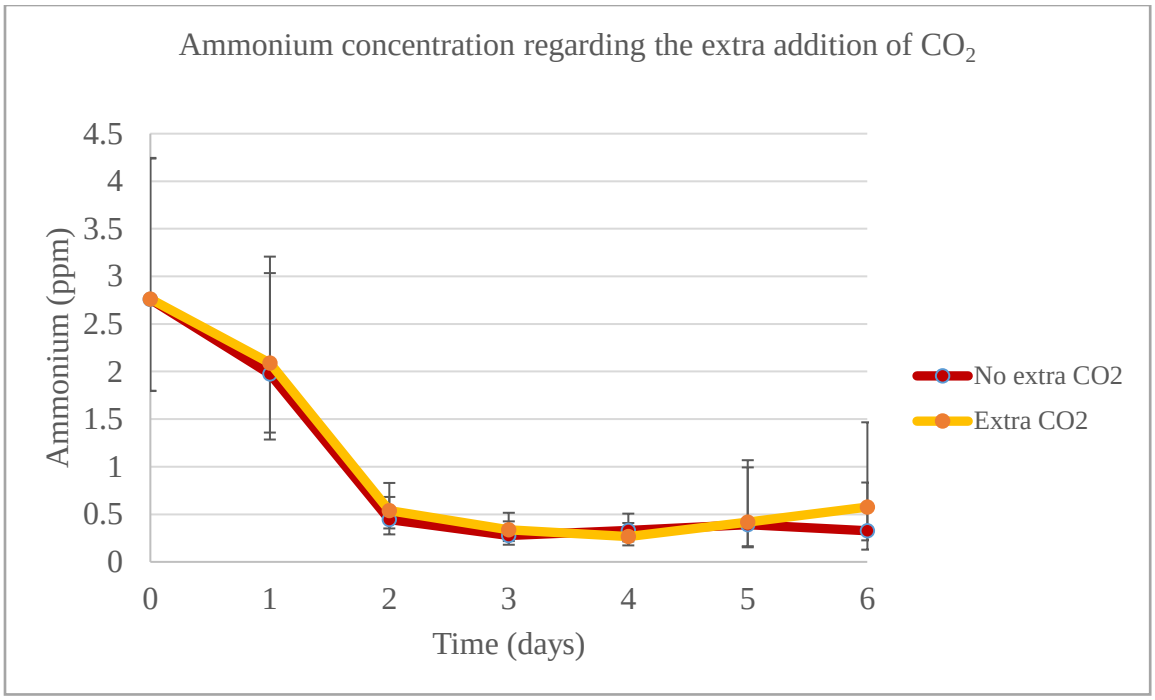

Figure 25. Ammonium concentration for the pilot-scale experiment when evaluating the significance of adding extra CO_2.

Table 29. Least square means for ammonium concentration in pilot-scale when evaluating the significance of adding extra CO_2 (n = 12).

	Estimate		Standard Error			
	Ratio		Lower		Upper	
Day	No	Yes	No	Yes	No	Yes
0	2.759	2.763	0.963	0.965	1.480	1.482
1	1.975	2.088	0.689	0.729	1.060	1.120
2	0.444	0.540	0.155	0.188	0.238	0.290
3	0.277	0.336	0.097	0.117	0.149	0.180
4	0.330	0.266	0.115	0.093	0.177	0.143
5	0.390	0.419	0.237	0.255	0.603	0.649
6	0.327	0.576	0.199	0.350	0.507	0.891

Table 30. Biochemical analysis of Chlorella sp. biomass produced using dairy effluent.

Analysis	Dry Weight (DW)	Units	Method
Protein	49.2	%	AOAC 990.03
Fat	2.32	%	AOAC 2003.05
Carbohydrates	38.5	%	Calculated
Fiber	6.6	%	AOCS Ba 6a-05
Neutral Detergent Fiber	32.8	%	Ankom Technology/AOAC 2001.11
Acid Detergent Fiber	11.6	%	ANKOM Tech. Method
Ash	9.91	%	AOAC 942.05
Total digestible nutrients	71.6	%	Calculated
Digestible energy	1.43	%	Calculated
Metabolizable energy	1.23	%	Calculated
Calcium	1.17	%	AOAC 985.01 (mod)
Phosphorus	1.70	%	AOAC 985.01 (mod)
Potassium	1.12	%	AOAC 985.01 (mod)
Sodium	0.48	%	AOAC 985.01 (mod)
Iron	5020	ppm	AOAC 985.01 (mod)
Manganese	157	ppm	AOAC 985.01 (mod)
Zinc	46	ppm	AOAC 985.01 (mod)

Chapter 4

Using Chlorella sp. from dairy wastewater in taste preference in pre-weaned dairy calves

Synopsis

Agricultural environmental problems may be caused by the excessive addition of nutrients due to livestock production. The objective of this study was to evaluate the taste preference of calves fed *Chlorella sp.* produced from dairy lagoon wastewater. *Chlorella sp.* biomass was produced outdoors using bioreactors. The biomass was sterilized and kept frozen at -4°C until the feeding trial. Six Holstein and crossbred dairy heifer calves were fed 0, 30, and 60 g of *Chlorella sp.* daily in a sequential elimination study. No difference were found for dry matter intake of calves fed 0, 30, or 60 g of *Chlorella sp.*, indicating that microalgae may be added to the rations of calves without any adverse effects.

4.1 Introduction

Microalgae has been used as a nutrition source for thousands of years by humans. However, large scale production of algae has grown over the last few decades (Görs, M. et al., 2010). In animal feeding, microalgae can provide essential nutrients, minerals, vitamins, fatty acids and a high protein content, which is difficult to find all in the same organism (Madeira, M. S. et al., 2017; Kotrbacek, V. et al., 2015; Christaki, E. et al., 2011). In this study, Chlorella sp. was used to recycle nutrients from a dairy wastewater lagoon at

the University of Minnesota West Central Research and Outreach Center (WCROC), Morris, Minnesota, and to produce high quality microalgae biomass to evaluate taste preference in dairy calves. It is important to note that this study closes a cycle, where the treated water can go back to the environment without been so harmful to it, while the nutrients kept by the microalgae can serve as feeding supplementation to cattle, with everything happening in the same farm.

Previous taste preference studies with different types of microalgae and macroalgae (Kuzmaite, I., et al., 2009; Erickson, P. S., et al., 2011; Heins, B. J. & Chester-Jones, H., 2015; Jeon, JY, et al., 2016; Schimek, D., et al., 2016) were conducted in the past. However, an integrating study involving the microalgae isolation from a dairy wastewater lagoon, using that microalgae to recycle the nutrients in the effluent, producing high-quality microalgae biomass, and conducting livestock feeding with the biomass produced has not been studied. Therefore, this study has the objective to produce a high nutritional feeding algae supplement and test the preference of dairy calves to a control calf starter compared to a calf starter supplemented with algae.

4.2 Materials and Methods

The microalgae used in this study was isolated from the dairy wastewater lagoon at the University of Minnesota West Central Research and Outreach Center in Morris, Minnesota. The microalgae biomass was produced using hanging bags bioreactor with *Chlorella sp*. to recycle the wastewater produced at the WCROC. The bioreactor's

structure was made using treated wood (height = 1.73m, length = 2.06m and width = 1.22m). Three pairs of flexible metal bars (diameter = 5mm, height = 1.34m), 0.03m from each other were installed to support the hanging bags shape. Plastic tubes (diameter = 15mm, height = 1.33m, length = 0.52m) were used to blow oxygen into the bags and add carbon dioxide when needed. The bottom of each plastic tube were drilled to open twelve holes (diameter = 0.5mm) for aeration.

The biomass was produced using different ratios of DW in water, in order to recycle nutrients from the dairy wastewater lagoon. Using an autoclave the *Chlorella sp*. biomass was sterilized under 121°C and at a pressure of 15 psi, for 30 minutes, in order not to be harmful to the calves involved in this study. After the sterilization, the biomass was kept frozen.

No mycotoxins were found in the biomass, including Aflatoxin (B1, B2, G1 and G2), DON (Vomitoxin), Fumonisin (B1, B2 and B3), Ochratoxin, T-2 toxin, and Zearalenone. Additionally, a heavy metals screening was conducted, and the levels of arsenic, mercury, cadmium, lead, and antimony, were below the maximum content recommended for animal feeding. Moreover, a detailed screening presenting the biochemical biomass content can be found in Table 1 conducted at the Midwest Laboratories®, Omaha - Nebraska.

All animal care and management for this specific study was approved by the University of Minnesota Institutional Animal Care and Use Committee (Animal Subjects Code number 1709-35098A). Six calves ranging in age from 12 to 14 weeks (106.71 ± 3.81 kg) old were enrolled in the taste preference study. The calves were used to test their preference for control, 30g/d, or 60g/day of *Chlorella sp.* produced *in situ* using dairy

wastewater. In this study, 2mm grains of *Chlorella sp.* were added to calf organic starter grain and hand mixed before feeding the calves.

The experiment was seven days (7d) long. Day one and day two were for adaptation (segment 1) and day three and day four were for data collection (segment 2). During the last three days (segment 3) of the experiment, the primarily consumed treatment was removed, and instead of feeding, the bucket was empty. The last three days were used to determine the second preferred treatment.

All six calves participated in the study at the same time. Calves were housed individually in hutches (2.12m x 1.14m x 1.22m) with outdoor access (17.98m^2) under solar panels (approximately 3m out of the ground), with free-choice water. Pinewood shavings were used as bedding inside and outside of the hutches.

Every morning, starting at 0800 h. the feed and orts were weighed using a hanging scale, and recorded. Five buckets (diameter = 28cm, height = 21cm) were placed outside of the hutches. The two buckets at the edges were used to avoid border effects, and the three buckets in the middle received 2.3 kg of feeding grain each. The treatments in each bucket were randomized during the whole experiment. The water bucket was allocated inside the hutch. The calves had five days to adapt to the new surrounds prior the beginning of this study, and they were fed as usual (1.16 kg/d) during this period.

Table 1 shows the nutritional difference between the three different calf starters. The analysis was conducted by the Rock River Laboratory. INC – Watertown, Wisconsin. The crude protein (CP) increased with the addition of *Chlorella sp.* in both treatments that contained the microalgae as a supplement. An increase up to 10.1% on CP content was

found when 60g of *Chlorella sp.* powder was added to 2300g of grain starter. A decrease in the fat content was found when 30g of the microalgae was added to the control, and the starch content decreased in both treatments with *Chlorella sp.* in relation to the control. Furthermore, there was a gradual increase in the mineral content when comparing the control with the treatments containing *Chlorella sp.*

Kendall's coefficient of concordance, W, was calculated to rank the consumption of the treatments from most to least preferred (Nombekela, et al., 1994) using JMP statistical (SAS Institute Inc.). Pairwise comparisons and Tukey adjustment were applied to evaluate the difference between the treatments total intake.

4.3 Results and Discussion

The total dry matter (DM) for the adaptation time (day one and two) for the different treatments is presented on Table 2. For this time period, the control was greatly consumed by the calves, followed by the control with 30g of *Chlorella sp.* and then the treatment with the highest content of microalgae, 60g.

The total dry matter intake (DMI) is in Table 3 for each one of the segments of the study. During the first segment the calves averaged 3.52 ± 1.38 kg of DM/d, 3.88 ± 1.31 kg of DM/d during the second segment, and 3.33 ± 1.87 kg of DM/d in the third segment. Neither for the segment two nor for segment three was the consumption different ($P > 0.05$).

Table 4 has DMI for three and four. The control treatment showed to be the first option on half of the time, being chosen six times out of twelve. The treatment where 30g of microalgae was added to the control was second and the 60g was third. Kendall's coefficient of concordance (W= 0.12) between control and 30g, and W=0.25 between control and 60g designated disagreement in preference rankings among the heifers in this study.

When ranking the data for the full experiment (Table 5), the control was the most preferred treatment. However, the results show that the control was the most preferred treatment for the calves that had the control plus 30g of microalgae as their first choice during the data collection time. Furthermore, the inverse is also true, since the control was the least preferred for the calves that had it as the first choice. Therefore, it was the treatment less consumed because they did not have control as a choice during the last three days of the experiment.

The control was preferred (1.36 times) by the calves the entire experimental time (7 days), but there was no significant difference between control and the other two treatments ($P > 0.05$). However, the difference between the control and the control with 30g of microalgae is not significant ($P > 0.05$). Furthermore, Table 7, shows the DMI per calf, indicating that the control was the first choice treatment for only two out of six calves participating in the study.

Previous studies with kelp (Erickson, P. S., et al, 2011; Heins, B. J. & Chester-Jones, H., 2015) showed that calves mainly preferred being fed without the addition of kelp to their grain starter. These results match the results presented in this study when considering total grain consumption. However, the studies with kelp showed that the calves

involved almost rejected the treatments with kelp. In this study, most of the calves had a treatment with *Chlorella sp*. as their first choice. Moreover, during the third segment, when the greater consumed treatment was removed, the DMI did not have much fluctuation, where the overall consumption per day was 3.33kg (Table 3).

The microalgae cellulosic cell wall is rigid and difficult to be digested, that is the main reason for the difficulty on substituting part or all the animal feeding for algal feeding (Kotrbacek, V. et al., 2015). Improvement on the digestibility was found when a mixture of *Chlorella* and *Scenedesmu sp.* was added to the milk fed to calves at roughly 10% of their body weights when compared to sesame seed oil (Chowdhury, S.A., et al., 1995).

Increasing protein on cattle feeding has a high economic cost, and it is also challenging to do it without decreasing or changing the amounts of minerals in the feeding. However, introducing dry feeding earlier (around three days of age) has been a practice well researched, and has demonstrated many benefits to the heifers. Considering the calves receptiveness to the taste of the *Chlorella sp*. biomass produced in this study, it is an interesting alternative to be tested in further studies where body changes (weight, size, and other body aspects) could be measured. Moreover, the addition of 60g of microalgae biomass in this study resulted in an increase of 10.1% in the protein content of the starter grain, suggesting that small amounts of *Chlorella sp*. would increase the CP rapidly in the feeding. The production of this microalgae is also an alternative for producers that want to treat dairy effluent and produce high protein feeding (the biomass produced during this study contained 49.2% CP, Table 1).

4.4 Conclusion

Previous studies with kelp showed adverse taste acceptance by calves and cows, indicating that even if the kelp has high nutrition values, the animals will not eat it because of the taste. However, the results presented in this study indicate that *Chlorella sp.* may be well accepted by calves when used as a supplement in their grain starter, providing a high protein, vitamins, minerals and less fat content when compared to other supplements. There was no difference in any of the segments of this study or the entire period of the study, indicating that the calves would eat any of the treatments without having a taste preference. Furthermore, this study shows that the *Chlorella sp*. biomass could be produced *in situ*, recycling nutrients from the effluent produced during the milking cycle, and help producers to decrease supplement feeding costs.

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Table 1. Nutrient composition in different treatment feeds offered to the calves.

Nutrient (% of DM)	Chlorella sp.	Control	30g of algae	60g of algae
Crude protein	49.2	18.60	18.95	20.48
Fat	2.32	6.88	6.39	6.96
Starch	38.5	47.10	43.36	40.49
Neutral detergent fiber	32.8	12.52	14.59	12.86
Acid detergent fiber	11.6	7.67	5.51	5.21
Ash	9.91	7.55	6.63	8.46
Calcium	1.17	0.92	0.90	1.15
Phosphorus	1.70	0.47	0.55	0.59
Potassium	1.12	0.78	0.80	0.87
Magnesium	157	0.18	0.18	0.21
Total digestible nutrients	71.6	-	-	-
Digestible energy	1.43	-	-	-
Metabolizable energy	1.23	-	-	-
Sodium	0.48	-	-	-
Iron	5020	-	-	-
Zinc	46	-	-	-

Table 2. Results dry matter intake for day one and two for the calf starters offered to calves.

	Treatment		
Heifer	Control	30 g of algae	60 g of algae
1	2.54	3.86	1.45
2	3.36	0.91	1.13
3	2.09	2.27	1.63
4	4.24	2.22	0.82
5	3.72	2.86	2.49
6	4.35	2.43	1.84
Total	20.30	14.54	9.37
Mean	3.38	2.42	1.56

Table 3. Average DMI in kg/d during the different segments of the experiment.

Segment	Day	Heifer						DMI/d
		1	2	3	4	5	6	
1	1 to 2	7.50	5.19	5.72	6.96	8.67	8.24	3.52
2	3 to 4	7.76	6.72	6.50	7.78	7.63	10.19	3.88
3	5 to 7	11.10	11.14	7.20	8.06	11.53	10.97	3.33
Mean[1]		8.79	7.68	6.47	7.60	9.28	9.80	

[1]Average consumption over all treatments in the three different segments

Table 4. Number of days that the treatment was chosen as first choice by the heifers during the collection data period, days three and four.

	Treatment		
Heifer	Control	30g of algae	60g of algae
1	1	0	1
2	2	0	0
3	0	1	1
4	1	1	0
5	1	1	0
6	1	1	0
Sum	6	4	2
Mean[1]	1.00	0.67	0.33

[1]Average per calf

Table 5. Ranking of dry matter intake from day 1 to 7 for overall calf starter consumption.

	Treatment		
Heifer	Control	30g of algae	60g of algae
1	3	1	2
2	3	2	1
3	1	2	3
4	1	2	3
5	1	3	2
6	1	3	2
Sum	10	13	13
Mean[1]	1.67	2.17	2.17

[1]The average closest to one means that the treatment was the most preferred, and the average closest to 3 means that the treatment was the least preferred.

Table 6. DMI for from day 1 through 7 during days 3 to 4 for calf starter grain.

	DMI (kg/d) from day 1 to 7			DMI (kg/d) from days 3 to 4		
Heifer	Control	30g of algae	60g of algae	Control	30g of algae	60g of algae
1	3.8	10.5	8.8	2.8	2.2	2.2
2	5.1	7.6	7.8	3.9	1.6	2.1
3	9.6	8.6	3.0	1.7	2.4	3.0
4	12.2	3.8	3.3	3.1	3.4	1.3
5	11.4	4.4	9.4	3.1	3.4	2.3
6	12.2	4.3	7.8	2.6	4.1	2.4
Sum	54.3	39.2	40.0	17.2	17.1	13.3
Mean*	9.1	6.5	6.7	2.9	2.9	2.2
Mean/d**	1.3	0.93	0.96	1.4	1.4	1.1

*Average per calf

**Average per calf per day

Chapter 5

Project Conclusion and Future Work

5.1 Problem and Solution Elucidation

Inefficient use of nitrogen and phosphorus leads to anthropogenic eutrophication of rivers, lakes, and oceanic basis worldwide, causing an environmental problem that can trigger a death cycle of an entire water body. Microalga, one of the organisms that benefit from the excessive nutrients runoff, and uses the sunlight to catalyze reactions that cause eutrophication can also be part of a solution. An integrative and practical solution has still not been realized, where the use of photobioreactors with microalgae could remove high nutrients amounts from wastewater and produce high protein biomass, to be used *in situ* or commercialized, as animal feed.

The study presented in this thesis represents the possible integrated solution for the nutrients accumulated - especially - in dairy farms, to be treated and used locally as a highly nutritious product for calves. To do that, plastic bags PBRs were installed beside a dairy barn, where reactions involving different dilutions of dairy wastewater in water were carried, aiming to find the best ratio where nutrients (N and P) could be removed from the effluent and biomass could be produced. Moreover, tests without the use of extra CO_2 were carried targeting to reduce treatment and production costs, while maintaining the high nutrients removal rates and biomass production. To close the cycle, calves from the same

farm that the wastewater was treated, were used in a taste preference study where the microalgae biomass produced was offered as a supplement mixed in their regular feeding.

5.2 Fulfilment of Project Aims

The project goals were listed as:

(i) Use a well-adapted microalgae strain to the Minnesota environment to conduct lab-scale experiments with different ratios of dairy wastewater, measuring nutrient removal rates, growth capacity and biomass productivity by the microalgae during lab-scale experiments;

(ii) Design a pilot-scale bioreactor and a monitor/controller system for pH and temperature;

(iii) Use the results from the lab-scale to conduct pilot-scale experiments with different ratios of dairy wastewater to optimize microalgae growth and nutrient removal rates in an outdoor bioreactor system. Measure nutrient removal rates, and growth capacity for biomass productivity by the microalgae during pilot-scale experiments;

(iv) Evaluate the necessity of adding CO_2 as an extra source of carbon and pH regulator to optimize microalgae growth;

(v) Conduct palatable livestock feeding studies in calves using *Chlorella sp.* biomass produced in the previous steps of this study

(i) Microalgae screening, identification and lab-scale experiments with different ratios of dairy wastewater

A screening with over five different species of microalgae isolated from a dairy wastewater lagoon in Morris, Minnesota – USA (45°35'38.0"N, 95°52'17.4"W), by researchers of the University of Minnesota. The microalgae that had the highest and faster growth rate was chosen, and it was identified as a *Chlorella sp.* using a ZR Fungal/Bacterial DNA MiniPrep (Catalog No. D6005) and primers developed by Dr. Brett Barney at the University of Minnesota, Twin Cities.

The lab-scale reactions occurred in cylinder glass tubes with a capacity of 1.2L each. The different ratios of dairy wastewater in water used were 1:10, 1:20, 1:30, and 1:40. Controls were also added to the experiment using AM6 medium. The influence of extra CO_2, pH, temperature, photosynthetic active radiation (PAR), were tested for the cell growth, biomass productivity and nutrients (NH_4^+, PO_4^{3-}, NO_3^-) removal. After seven days of experiment, the highest biomass productivity were achieved by ratio 1:10 and followed by the control, 1.575 ± 0.599 g/L and 1.315 ± 0.240 g/L, respectively.

Considering the nutrients removal rates, 1:30 removed up to 97.39% of the phosphate, followed by 1:20 with 96.86%. Control removed the highest amount of nitrate, 97.03%, followed by 1:10, which removed 74.96%. The ammonium concentration dropped significantly in all the different treatments (over 95%), but 1:10 could remove up to 99.53% of the NH_4^+ content.

(ii) Design a pilot-scale bioreactor and a monitor/controller system for pH and temperature

The pilot-scale bioreactors were designed to have six bags in one system, where all of them could receive approximately the same amount of solar radiation during the day. The structure was made using treated pinewood (height = 1.73m, length = 2.06m and width = 1.22m). Three pars of flexible metal bars (diameter = 0.05m, height = 1.34m), distant 0.03m from each other, were installed to support the shape of the hanging bags. PVC tubes (diameter = 0.015m, height = 1.33m, length = 0.52m) were used to blow oxygen into the bags and carbon dioxide, when needed. At the bottom of each PVC tube, twelve holes (diameter = 0.5mm) were drilled for the air to flow, aerating and mixing the whole system. The airflow was measured using an 8360 VelociCalc® Plus from TSI, and it was flowing in a rate of 4.61 ± 0.09 m^3/min from the blower into the bag through the larger black hose (this rate was measured in each one of the bags individually). A system called Apex Fusion® was added to measure and record temperature and pH changes, and also regulate automatically (after some modifications and additions) the pH by adding CO_2 from a liquid CO_2 tank.

(iii) Pilot-scale tests to measure growth capacity for biomass productivity, and nutrient removal rates

No significant difference ($P < 0.05$) on cell density was registered at day 0 for any of the treatments, meaning that the bags had a similar number of cells/L at

each of the three different batches in this experiment. Control produced an estimate of 0.64g/L (0.39 -1.05g/L) of biomass, while 1:10 (DW in water) produced 0.33g/L (0.22-0.50 g/L) in the last day. Day (0-6), ratio, and the interaction between day and ratio were significant variables for biomass productivity. In this case, biomass productivity is dependent on the pH and solar radiation. For the ratios that were harvested on Day 7, pH and solar radiation were not significant, and the only variable significant for the model was ratio.

When analyzing the phosphate concentration, controls were significantly different from all the ratios every day of the experiment because the AM6 medium contains a high amount of prosperous in its recipe. The ratios were not significantly different from each other on any day. Coincidentally with the last day of the log phase for cell growth on ratios 1:10, 1:30, and 1:40, the day with less amount of phosphate dissolved in the DW/water was day 4.

All the treatments harvested had very high removal rates for ammonium, dropping the levels close to zero in all three different weeks. Control removed 98.12%, while the ratios 1:10 and 1:30 removed 97.55% and 95.53%, respectively. Day (0 - 6) and ratio were significant variables for Ammonium removal, and it was dependent on solar radiation. For the ratios harvested on day 7, solar radiation was not significant, and ratio was the only significant effect for the model.

The highest nitrate removal rates in pilot-scale were observed for the control (84.11%), 1:10 (39.27%), and 1:20 (34.96%), at the seventh day of the experiment. Day (0 - 6), ratio, and the interaction between day and ratio had an effect on nitrate

removal rates. For the ratios harvested on day 7, ratio was the only variable significant for the model.

(vi) Evaluating the necessity of adding CO_2 as an extra source of carbon and pH regulator to optimize microalgae growth;

For this experiment, the treatments used were control, control N[2], 1:10, 1:10N, 1:30, and 1:30N.

In lab-scale the proportion of biomass production in the treatments with extra CO_2 is higher when placing the three treatments together, being significant different in the last two days of experiment (5 and 6). However, between day 0 and 4 no significant difference was observed in the biomass productivity regarding the extra CO_2 addition. The higher phosphate removal rate was observed in the ratios 1:30 (up to 96.11% for 1:30 and as high as 98.61% for 1:30N). When considering only the extra addition or not of CO_2, the tubes that did not receive carbon dioxide had a significant lower concentration of PO_4^{3-} than the tubes with extra amounts of CO_2. The tubes with extra CO_2 seemed to have higher nitrate removal rates in the final day, but the tubes without extra gas removed higher rates throughout the experiment, 78.66% without extra CO_2 and 75.18% with extra CO_2. On the last experimental day, the dilutions without extra CO_2 removed up to 96.56% of the initial ammonium concentration, while the tubes with extra CO_2 addition removed up to 97.74%

[2] N means that no extra CO_2 was added to the treatment.

Throughout pilot-scale, the formation of biofilm in the bag's walls, which did not allow the proper sampling for cells and biomass findings. Because of that, when the harvest occurred for each one of the bags, the higher biomass weight was harvested from the control, and the amount collected was significantly different from the ratios 1:10 and 1:30 in the same conditions. However, after harvesting the bags where the extra CO_2 was not added, there was no significant difference between the controlN and 1:10N, but both of them were different from the ratio 1:30N in biomass productivity. The maximum phosphate removal rates were registered on day 4 for all the bags, having 1:30 as the ratio with higher removal rates, either with extra CO_2 (98.29%) or not (93.05%), followed by 1:10 (82.80%), 1:10N (72.11%), and the controls (45.68% for control and 15.79% for controlN). The extra addition of CO_2 was not a significant effect in any day of the experiment for PO_4^{3-}. The controls removed the maximum nitrate amounts on the last day of the experiment. During this experiment, the ratio 1:10N had a removal rate 69.78% in the last day, and the control removed 73.66% on day 6. Furthermore, there was no significant difference between the ratios and the extra addition of CO_2 in any day of this experiment regarding the nitrate removal rates, but overall the treatments with the additional amount of the gas removed higher rates of nitrate. The controlN had the highest ammonium removal rate, 94.47%, followed by the ratio 1:10N, which removed up to 92.19% throughout the experiment. The bags with an extra addition of CO_2 showed to overall remove less ammonium, but adding extra CO_2 showed to not be a significant effect in any of the experimental days for NH_4^+ removal.

(vii) Conducting palatable livestock feeding studies in calves using *Chlorella sp.* biomass produced in the previous steps of this study

Sterilized biomass was used for feeding trials with six Holstein and crossbred dairy heifer calves. No mycotoxins were found in the biomass and many heavy metals were tested, having the levels below the maximum content recommended for animal feeding. The microalgae biomass produced had a protein content of 43.2%, 2.32% of fat, 38.5% of carbohydrates, and around 10% of different minerals and nutrients. They were fed 0, 30, and 60 g of *Chlorella sp.* daily in a sequential elimination study. No difference were found for dry matter intake of calves fed 0, 30, or 60 g of *Chlorella sp.*, indicating that microalgae may be added to the rations of calves without any adverse effects.

5.3 Future work

Considering the high amount of phosphate (up to 72.11%), ammonium (up to 92.19%), nitrate (up to 69.78%), and the promising *Chlorella sp.* biomass production for ratio 1:10N, this treatment seems to be a good option to be used in further tests. To keep the reactions going is to build a PBR continuous system, where dairy wastewater could be added every four or five days (when the cells start to deplete in number) to give the microalgae more nutrients, but the treated effluent could be removed before the new

addition. It would also be valuable to add another treatment step, like a hydroponic system, for example.

Microalgae biomass harvesting techniques need to get more straightforward and faster. It was noticed in experiments that were not reported in this thesis, that longer batches where more nutrients were added weekly, produced larger, stronger and greener microalgae cells (harvested after two or three weeks from day 0). During this more extended experiments, the harvesting was easier because the cells were settling down in less than two hours after turning off the air blower.

Longer reactions times and more accessible harvesting techniques will help producers to apply this kind of technology in their own farms. The study presented in this thesis produced a biomass 49.2% rich in protein, which makes it an excellent option for animal feeding, and very cheap since it was a secondary product of the wastewater treatment. Moreover, most of the calves exposed to ration supplement with *Chlorella sp.* were positive in the taste preference tests. Further studies, including more prolonged exposure and blood tests, can be done to help to understand the role of the *Chlorella sp.* in calves' or other ruminants' health.

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